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Adenovirus vector-mediated Gli1 siRNA induces growth inhibition and apoptosis in human pancreatic cancer with Smo-dependent or Smo-independent Hh pathway activation *in vitro* and *in vivo*

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ABSTRACT

Activation of Hedgehog (Hh) signaling pathway is a core molecular mechanism in pancreatic carcinogenesis. However, the inhibition of upstream Hh signals does not inhibit the growth of a subset of pancreatic cancer (PC). This study was to examine the effect of siRNA targeting Gli1, the downstream component of Hh pathway, on PC cells and to provide some insight into the underlying mechanisms. A Gli1siRNA-expressing adenovirus (Ad-U6-Gli1siRNA) was constructed, and its effect on PC cells was investigated *in vitro* and *in vivo*. Gli1 was expressed in 83.3% (20/24) PC tissues, whereas no expression was found in normal pancreatic ductal epithelium. Gli1 was expressed in SW1990 and CFPAC cells in which Smo was completely absent, as well as in PaTu8988, Panc-1 and BxPC-3 cells in which Smo was concomitantly present. Ad-U6-Gli1siRNA induced cell growth inhibition, strong G0/G1 cell cycle arrest and apoptosis in all five human PC cell lines. Meanwhile, Ad-U6-Gli1siRNA significantly suppressed the expression of Gli1, Ptch1 and two target genes, Cyclin D2 and Bcl-2, in all five lines. Furthermore, two tumor xenograft nude mice models were established by subcutaneously injecting Smo-positive Panc-1 cells or Smo-negative SW1990 cells. The *in vivo* experimental results demonstrated that Ad-U6-Gli1siRNA inhibited the growth of both Panc1-derived and SW1990-derived tumors and induced cell apoptosis. Our study indicates that Gli1-targeting siRNA could induce growth inhibition and apoptosis in PC through knockdown of Gli1 and its target genes; and this method may represent a more effective therapeutic strategy for PC with Smo-dependent or Smo-independent Hh pathway activation.

1. Introduction

Pancreatic cancer (PC) is one of the most lethal malignancies that has not yet been successfully controlled, despite impressive improvements in surgical and chemotherapeutic approaches [1]. Aberrant activation of Hedgehog (Hh) pathway has been implicated in the growth of PC [2–4]. Hh signal transduction is initiated by the binding of Hh protein (Sonic, Indian and Desert Hh) to the 12-pass transmembrane domain receptor Patched (Ptch), which abolishes the inhibitory effect of Ptch on the 7-pass transmembrane domain receptor Smoothened (Smo) and consequently allows Smo to induce nuclear translocation of Gli transcription factors, which activate the transcription of target genes, including Ptch1 [5,6]. Constitutive activation of the canonical Hh/Gli signaling pathway, in which either the Hh ligand or receptor is necessary for inducing Gli activation, has been shown to play a functionally important role in the genesis and development of PC [7,8]. Given the Hh pathway's critical role in PC, specific agents targeting core components of Hh pathway, such as Shh antibody and Smo antagonists, have been developed for combating PC. However, these agents have been proven to be reasonable yet limited with disappointing results, because a subset of PC has been shown to be resistant to the treatment of these agents [9–11].

Recently, emerging evidence has proposed a noncanonical Hh signaling pathway, in which Gli activation could be driven through Smo-independent mechanisms [11,12]. The results of our previous study showed that Gli1 was consistently present in two Smo-negative PC cell lines, SW1990 and CFPAC, and that these two lines were not inhibited by the treatment of antisense Smo delivered by an adenoviral vector, indicating that Gli1 activation could be regulated independently of Smo-mediated signaling and result in resistance of PC cells [13]. Accumulating studies have suggested that in many malignancies, several other pathways, including mTOR/S6K1, PI3 K/AKT and RAS/RAF/MEK/ERK signaling, could activate the Gli code by interacting with Hh pathway downstream of Smo [14–17]. Additionally, one study reported that Kindlin-2 could promote Gli1 expression through a Smo-independent mech-

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anism involving GSK3β inactivation [18]. Another study reported that Kras and TGF-β could induce Gli expression in Smo-negative PC cells and thus result in cell survival and Kras-mediated cellular transformation [19]. Taken together, these results may explain the failure of the aforementioned agents which act at the level of Smo or above that only tumors in which Hh pathway activation occurs upstream or at the level of Smo could be treated by these agents, whereas tumors arising from activation of Hh pathway downstream of Smo will most likely not respond to them. Therefore, more effective strategies against PC based on blockade of Hh pathway are urgently needed, and better identification of potentially targeted components downstream of Smo is desperately required.

Table 1
Primers used for quantitative real time RT-PCR.

Gene	RT primer	Annealing temperature
Gli1	Sense: TGTGTATGAAACTGACTGCCG Antisense: CCCAGTGGCACACGAACTC	55 °C
Ptch1	Sense: TGTGCGCTGTCTTCCTTCTG Antisense: CACGGCACTGAGCTTGATTC	55 °C
Cyclin D2	Sense: TGCTACTATGAAGACCGCCAAT Antisense: CTTCCACCAGCTCCACATTC	55 °C
Bcl-2	Sense: CATGCCAAGAGGGAAACACCAGAA Antisense: GTGCTTTGCATTCTTGGATGAGGG	55 °C
β-actin	Sense: AGAGCTACGAGCTGCCTGAC Antisense: AGCACTGTGTTGGCGTACAG	55 °C

Gli transcription factors, functioning as the final and essential effectors of Hh pathway and the integrative platform of numerous signaling inputs, play a key role in promoting tumor growth and metastasis. Among three Glis, Gli1 is the strongest transcriptional activator that directly regulates the transcription of target genes. Gli1 expression has been reported to be the only loyal marker of Hh pathway activity [20]. Thus, blocking Gli1 at the distal end of Hh pathway is anticipated as a more promising strategy for combating a wide spectrum of PC with Hh pathway activation occurring at different nodal points.

To examine whether Gli1 inhibition was more efficient at treating PC, Gli1 siRNA delivered by an adenoviral vector (Ad-U6-Gli1siRNA) was engineered, and its effect on PC cells, including Smo-negative SW1990 and CFPAC cells, was analyzed. Moreover, the *in vivo* antitumor efficacy of Ad-U6-Gli1siRNA was evaluated using two xenograft tumor models individually derived from Smo-positive Panc-1 and Smo-negative SW1990 cells. Our results showed that suppression of Gli1 expression induced growth inhibition and cell apoptosis in PC with either Smo-dependent or Smo-independent Hh pathway activation, *in vitro* and *in vivo*, suggesting that Gli1 siRNA could be used as a preferential agent for treating PC.

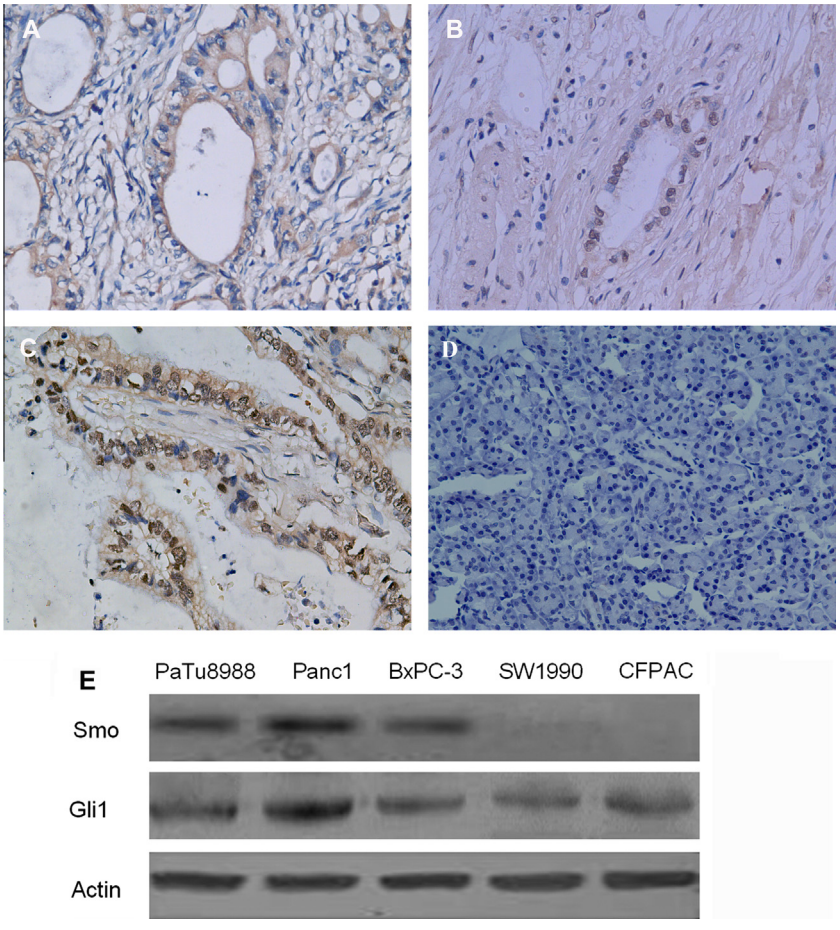

Fig. 1. Expression of Gli1 protein in PC tissues and in five human PC cell lines. (A–D) Immunohistochemical analysis of Gli1 protein in PC tissues and normal pancreatic samples by using a polyclonal rabbit anti-human Gli1 antibody. The positive signal in the cytoplasm (A), nucleus (B) and both the cytoplasm and nucleus (C). No positive signal in normal pancreatic ductal cells (D). (E) Western blot analysis of Gli1 protein expression in five human PC cell lines. Cells were collected and lysed with RIPA lysis buffer. Equal amounts of proteins were separated on SDS–PAGE gels and electrotransferred to nitrocellulose membranes at 4 °C for 2 h. Membranes were probed with primary antibody at 1:100 dilution overnight at 4 °C and then incubated with secondary antibody. Proteins were detected by enhanced chemiluminescence with β-actin serving as loading control. For A–D, magnification is 400×.