GLI2 Knockdown Using an Antisense Oligonucleotide Induces Apoptosis and Chemosensitizes Cells to Paclitaxel in Androgen-Independent Prostate Cancer

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Abstract

Purpose: GLI transcription factors mediate hedgehog signaling and have been implicated in several human malignancies, including prostate cancer. The objectives of this study were to characterize GLI2 expression levels in human prostate cancer cell lines and tissues to test the effect of antisense oligonucleotide (ASO) targeting GLI2 on androgen-independent (AI) prostate cancer cell lines.

Experimental Design: A tissue microarray was used to characterize differences in GLI2 expression in benign prostate hyperplasia, prostate cancer treated by neoadjuvant hormonal therapy and AI prostate cancer. The effects of GLI2 ASO on PC-3 cell growth and paclitaxel chemosensitivity were assessed in vitro and in vivo. Oligonucleotide spotted microarray analysis was used to determine alteration in GLI2 coregulated genes after ASO treatment.

Results: The expression of GLI2 was significantly higher in prostate cancer than in benign prostate hyperplasia, decreased after androgen ablation in a time-dependent fashion, but became highly expressed again in AI prostate cancer. GLI2 ASO treatment of PC-3 cells reduced GLI2 mRNA and protein levels in a dose-dependent manner. GLI2 knockdown increased PC-3 cell apoptotic rates and significantly decreased cell growth and modulated levels of apoptosis-related genes, such as Bcl2, Bcl-xL, and clusterin. GLI2 knockdown also changed levels of several cell cycle regulators, such as cyclin D1, p27, and PKC-δ. Systematic administration of GLI2 ASO in athymic mice significantly delayed PC-3 tumor progression and enhanced paclitaxel chemosensitivity.

Conclusions: These findings suggest that increased levels of GLI2 correlates with AI progression and that GLI2 may be a therapeutic target in castrate-resistant prostate cancer.

Prostate cancer is the most common cancer in men and a leading cause of death from cancer in North America (1). Androgen ablation and chemotherapy, the only therapies that have been shown to prolong survival, only induce short-term remissions because surviving tumor cells usually recur with an androgen-independent and chemoresistant phenotype with the eventual progression of disease (2–4). Development of advanced disease is a complex process involving up-regulation of antiapoptotic survival genes and activated growth factor and signaling pathways. To significantly improve survival in men with prostate cancer, new therapeutic strategies designed to inhibit the emergence of this phenotype must be developed.

The sonic hedgehog (SHH) signaling pathway is integral in human organ embryogenesis, and recently, activation of this pathway in adults has been implicated in many different tumor types, including skin (5), breast (6), lung (7), pancreatic (8), stomach (9), liver (10), and prostate cancers (11–13). SHH is one of three hedgehog ligands in humans that bind the transmembrane receptor Patched1 (Ptc1). Ptc1 constitutively inhibits a second transmembrane protein Smoothened (Smo). After SHH binds to Ptc1, inhibition of Smo is lost, which leads to the activation of a group of transcription factors, including GLI1, GLI2, and GLI3. GLI1 and GLI2 are transcriptional activators of hedgehog target genes, whereas GLI3 acts mainly as a repressor (14–16). Although normally dormant in adult prostate, recent evidence indicate that the SHH-GLI pathway is activated in human prostate cancers when compared with normal prostate epithelium (12, 13, 17). In situ hybridization and quantitative reverse transcription–PCR (RT-PCR) of prostatectomy specimens indicate that cancers have increased expression of markers of SHH-GLI pathway activation, including SHH, GLI1, and Ptc1, compared with normal epithelium.

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GLI2 seems to be the major nuclear effector of hedgehog signaling (18, 19), and the role in carcinogenesis has been shown in the context of several cancers (20–22). In prostate cancer, GLI2 protein has been reported to be highly expressed in prostate cancer cell lines and human prostate cancer tissues (23).

In this study, we further characterized the relationship between GLI2 expression and human prostate cancer in men receiving hormone therapy, as well as hormone refractory tumors. In addition, we tested the effect of antisense oligonucleotide (ASO)‐induced GLI2 knockdown on androgen‐independent (AI) prostate cancer cell lines.

Materials and Methods

**Tumor cell lines and reagents.** The human prostate cancer cell lines PC-3 and DU145 were purchased from the American Type Culture Collection and maintained in DMEM (Invitrogen-Life Technologies, Inc.) supplemented with 5% fetal bovine serum. LCNAp and C4-2 cells were kindly provided by Dr. Leland W.K. Chung (Emory University) and maintained in RPMI 1640 (Invitrogen) supplemented with 5% fetal bovine serum.

**Chemotherapeutic agents.** Paclitaxel was purchased from Bionyx Pharma (St. Catharines). Dr. Helen M. Burt (Pharmaceutical Science, University of British Columbia) generously supplied the polymeric micellar paclitaxel used for in vivo studies. Stock solutions of paclitaxel were prepared in PBS to the required concentrations before each in vitro or in vivo experiment.

**Treatment of cells with GLI2 ASO.** The GLI2 ASO used in this study was modified at the 2’ position of the sugar with a 2’-O-(methoxymethyl) linkage. The sequence of GLI2 ASO was 5’-GTGGCGGCACTCCAGCGAGG-3’. The control oligonucleotide (control ODN) used was designed not to match any mRNA in the human or mouse transcriptomes and has the sequence 5’-CCITCCCTGAAAGCTTCCTCC-3’. Cells were plated at the density of 4,000 cell/cm² and treated a day later for two successive days with ASO. Oligofectamine (Invitrogen), a cationic lipid, was used as a transfection reagent. PC-3 cells were treated with various concentrations of ASO in a manner described previously (24).

**Northern blot analysis.** Total RNA was isolated from cultured PC-3, DU145, LCNAp, and C4-2 cells using the TRIzol Reagent (Invitrogen). Electrophoresis, hybridization, and washing conditions were carried out, as reported previously (24, 25). Briefly, 20 µg of total RNA from each sample were subjected to electrophoresis on 1.0% agarose/formaldehyde gels and transferred to nylon membranes overnight at room temperature according to standard procedures. Human GLI2 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) complementary DNA probes were generated by RT-PCR from RNA of DU145 or LCNAp cells using primers 5’-TCAGACTTACCCACACGATGC-3’ (sense) or 5’-AGATGAGGTTTCTCGTGGATG-3’ (antisense) for GLI2 and primer 5’-TGCTTTTAACTCTGGTAAAGT-3’ (sense) and 5’-ATATGTGG-CAGGTTTCTAGA-3’ (antisense) for GAPDH. The RNA blots were hybridized overnight with the human GLI2 probe labeled with [32P]CTP by random primer labeling. The RNA blots were exposed to film after washing. After the detection of GLI2 expression, the membranes were reprobed with human GAPDH to verify the integrity of the RNA.

**Quantitative RT-PCR.** To quantify the expression of GLI2 mRNA, quantitative RT-PCR was done. The same RNAs used for Northern blot analysis were used for quantification. After treating total RNA with DNaseI (Invitrogen), total RNA (2 µg) was reversed transcribed using random hexamers (Perkin-Elmer Applied Biosystems) and 20 units of Molony murine leukemia virus reverse transcriptase (Invitrogen) in a total volume of 30 µl at 25°C for 10 min, followed at 37°C for 1 h and denaturation at 95°C for 5 min. The ABI PRISM 7900HT Sequence Detection System (Applied Biosystems) was used for real-time monitoring of PCR amplification of complementary DNA. Complementary DNA samples were mixed with the Taqman Universal PCR Master Mix (Applied Biosystems). Relative quantification of gene expression was done as described in the manual using RNA as an internal standard. For GLI2 amplification, Taqman Gene Expression Assay reagent (Hs00257977_m1) was purchased from Applied Biosystems. For β2 amplification, the primers and Taqman probes described previously were used (26). The thermal cycling conditions were 50°C for 2 min, 95°C for 15 s, and 60°C for 1 min. The comparative cycle threshold (Ct) method was used for relative quantification of target mRNAs. Each assay was done in triplicate.

**Western blot analysis.** Samples containing equal amounts of protein (depends on the antibody, 20-100 µg) from lysates of cultured PC-3 cells underwent electrophoresis on a SDS-polyacrylamide gel and were transferred to nitrocellulose filters. The filters were blocked in Odyssey Blocking Buffer (LI-COR Biosciences) at 4°C for 1 h and then incubated overnight with a 1:5,000 dilution of antihuman GLI2 rabbit polyclonal antibody (Abcam, Inc.) at 4°C and for 1 h with 1:10,000 diluted antihuman Vinculin mouse monoclonal antibody (Sigma Chemical Co.) at room temperature with gentle shaking and washed thrice with washing buffer (PBS containing 0.1% Tween 20) for 5 min. Filters were then incubated for 1 h with 1:5,000 diluted Alexa Fluor 680 goat anti-rabbit and anti-mouse antibodies (Invitrogen) at room temperature. Specific proteins were detected using ODYSSEY IR imaging system (LI-COR Biosciences) after washing, as described above. The monoclonal and polyclonal antibodies (Bel2, Bel-c1, Mcl-1, clusterin, cyclin D1, cyclin E2, p27, PKC-γ) were purchased from Santa Cruz Biotechnology for screening of the genes related with GLI2 and apoptosis assessment. The anti-GLI1 polyclonal antibody was purchased from Abcam, and the anti–polyl(ADP-ribose) polymerase and anti–phosphorylated Rb antibody was obtained from Cell Signaling Technology.

**Neoadjuvant hormone therapy tissue microarray.** Slides (H&E) containing tissue samples from 112 radical prostatectomy specimens (collected between 1989 and 2003) were obtained from the Vancouver General Hospital. Fourteen AI patients were obtained from metastatic lesions (bone, lymph node, liver, and adrenal) of the warm autopsy at the University of Washington. All AI patients had progressive prostate cancer after either chemical or surgical castration, both clinically and with rising PSA, and died of prostate cancer. Supplementary Table S1 shows the treatment groups. Benign and cancer sites were identified and marked in donor paraffin blocks using matching H&E reference slides. Tissue microarray was constructed using a manual tissue microarrayer (Beecher Instruments). Each marked block was sampled thrice with a core diameter of 0.6 mm arrayed in a rectangular pattern with 1 mm between the centers of each core, creating a triplicate tissue microarray layout and ordered by treatment of the patients (Supplementary Table S1). The 126 patient specimens were spotted in triplicate to create a tissue microarray with 378 cores. The tissue microarray paraffin block was sectioned into 0.5-µm sections and mounted on the positively charged slides. Using rabbit polyclonal antibody against GLI2 (Abcam), immunohistochemical staining was conducted by Ventana autostainer model Discover XT (Ventana Medical System) with enzyme labeled biotin streptavidin system and solvent resistant 3,3'-diaminobenzidine Map kit. Slides were mounted with coverslips using the xylene-based mounting medium, Cytoseal (Stephan Scientific). Using identical microscopic and camera settings [reverse transcription color-SPOT high-resolution digital camera (Diagnostic Instruments) mounted on an Olympus System light microscope (model BX51)], digital images were taken from representative areas reflecting the overall staining to strong staining by a pathologist (L. F.). The staining intensity was evaluated and scored by a pathologist (L. F.). All comparisons of staining intensities and percentages were made at 200× magnifications.
In vitro cell growth assays. Cell growth of PC-3 cells was assessed using crystal violet assay, as described previously (27). Cells were plated in 12-well plates, allowed to attach 24 h, and treated once daily with various concentrations of ASO for 2 d. The absorbance was determined with a microculture plate reader (Becton Dickinson Labware) at 560 nm. Absorbance values were normalized to the values obtained from the vehicle-treated cells to determine the percentage of survival. To assess the combination of GLI2 ASO and paclitaxel in vitro, paclitaxel was added at various concentrations (0-10 nmol/L) after GLI2 ASO treatment. Cells treated with this combination treatment were cultured 2 or 4 d after transfection, and then the crystal violet assay was carried out.

Flow cytometric analysis. Flow cytometric analysis of propidium iodide–stained nuclei was done, as described previously (28). In brief, cells were plated in 10-cm² dishes and, on the day after, were treated as described above. The cells were trypsinized 2 d after ASO treatment, fixed in 70% ethanol overnight at 4°C, then incubated with 1 µg/mL RNase (Sigma) for 30 min at 37°C before stained with 5 µg/mL propidium iodide (Sigma) for 1 h at room temperature. The stained cells were analyzed for relative DNA content on a dual laser flow cytometer (Beckman Coulter Epics Elite; Beckman, Inc.). Each assay was done in triplicate.

Microarray procedure, data processing, and analysis. Total RNA from the PC-3 cell line was obtained 2 d after transfection with GLI2 ASO and control ASO (250 nmol/L). Three independent transfections were done to minimize experimental variation. Total RNA from each treatment sample (GLI2 ASO, 250 nmol/L) was compared with its control (250 nmol/L) on the same microarray. A dye swap for

Fig. 1. Expression of GLI2 in prostate cancer cell lines and human prostate tissues. A, total cellular RNA was extracted from parental prostate cancer lines, and GLI2 and GAPDH mRNA expression was analyzed by Northern blotting. B, quantitative analysis of GLI2 mRNA levels was analyzed by quantitative RT-PCR and average GLI2 mRNA levels were normalized to levels of rRNA. Asterisks indicate significant difference between PC-3 and other prostate cancer cell lines (*, P < 0.05). C, GLI2 immunostaining in a human prostate tissue microarray. Lower magnification (a–f), higher magnification (g–l). a and g, benign prostate hyperplasia (BPH); b and h, neoplastic glands in untreated tumors (0-NHT); c, i and d, j, prostate cancer after NHT (3M and 3-6M, respectively), illustrating decreased immunoreactivity; e and k, illustrating strong immunoreactivity after 6 mo after NHT; f and l, hormone refractory prostate cancer showing highly intensive and uniformly immunostaining. D, data from 126 samples were calculated. Columns, mean; bars, SE. Mean GLI2 staining in benign prostate hyperplasia, NHT, and AI tissues. Specimens were graded from 0 to +3 representing the range from no staining to heavy staining by visual scoring. Immunoreactivity of GLI2 is significantly higher in both untreated NHT and AI group compared with benign prostate hyperplasia (#, P < 0.05). After short-term androgen ablation (6 mo), GLI2 staining is significantly reduced (*, P ≤ 0.05; **, P ≤ 0.01), but increases again after longer-duration androgen ablation (+6 mo).
each pair was done to account for dye bias. Microarrays of 21,000 (70-mer) human oligonucleotides representing 21,000 genes (Operon Technologies) printed in duplicate in 3 x SSC onto aminosilane-coated slides (ERIE C28) were supplied by the Array Facility of The Prostate Center at Vancouver General Hospital. Arrays were hybridized with 3DNA DNA Dendrimer Probes generated from 10 μg total RNA according to manufacturers' directions (Genisphere). Briefly, reverse transcription incorporated a specific sequence present on the 5' end of the reverse transcription primer supplied with the kit. The complementary DNA was hybridized to the array overnight at 42°C. After stringent washes, the fluorescent 3DNA reagent, which includes a "capture sequence" complementary to the sequence at the 5' end of the reverse transcription primer, was hybridized to the complementary DNA (47°C for 2-3 h). After further washes, the arrays were immediately scanned on a ScanArrayExpress Microarray Scanner (PerkinElmer). Signal quantity and quality were assessed using Imagene 5.6 (BioDiscovery). Data from Imagene were analyzed in GeneSpring 7.2 (Silicon Genetics) for profiling changes in gene expression. Analyses done in GeneSpring include background correction, LOWESS normalization and hierarchical clustering using standard correlation. Differentially expressed genes were identified by two-sample t tests, and P values were adjusted for multiple comparisons using the false discovery rate method of Benjamini and Hochberg. The Ingenuity pathway analysis (Mountain View, CA) was used to examine functional and network associations between the complementary DNA (47°C for 2-3 h). After further washes, the arrays were immediately scanned on a ScanArrayExpress Microarray Scanner (PerkinElmer). Signal quality and quantity were assessed using Imagene 5.6 (BioDiscovery). Data from Imagene were analyzed in GeneSpring 7.2 (Silicon Genetics) for profiling changes in gene expression. Analyses done in GeneSpring include background correction, LOWESS normalization and hierarchical clustering using standard correlation. Differentially expressed genes were identified by two-sample t tests, and P values were adjusted for multiple comparisons using the false discovery rate method of Benjamini and Hochberg. The Ingenuity pathway analysis (Mountain View, CA) was used to examine functional and network associations between differentially expressed genes. Significances for biological functions compared with the whole Ingenuity pathway knowledge base as a reference set.

Assessment of in vivo tumor growth. Approximately 2 x 10⁶ PC-3 cells were inoculated s.c. in flank region of 6-wk-old to 8-wk-old athymic nude mice (Harlan Sprague-Dawley, Inc.) via a 27-gauge needle under isoflurane anesthesia. When PC-3 tumors reach 200 mm³ (usually 3-4 wk after injection), mice were randomly selected for treatment with GLI2 ASO alone, control ODN alone, GLI2 ASO plus paclitaxel, and control ODN plus paclitaxel. Each experimental group consisted of 9 or 10 mice. After randomization, 15 mg/kg GLI2 or control ODN was injected i.p. thrice a week for 8 wk for ASO monotherapy and for 9 wk in the ASO plus paclitaxel groups. A total of 0.5 mg micellar paclitaxel was given i.v. thrice per week from days 7 to 14 and from days 21 to 28. Tumor volume measurements were done once weekly and calculated by the formula: length x width x depth/0.5236 (29). Data points were expressed as average tumor volume levels ± SE. All animal procedures were done according to the guidelines of the Canadian Council on Animal Care and with appropriate institutional certification.

Statistical analysis. All statistical analyses were done using Microsoft Excel with Statcel 2 software. All results were expressed as the mean ± SE and significance of differences were by Student’s t test or ANOVA repeated measurement. P values were adjusted for multiple comparisons using the false discovery rate method of Benjamini and Hochberg. The Ingenuity pathway analysis (Mountain View, CA) was used to examine functional and network associations between differentially expressed genes. Significances for biological functions compared with the whole Ingenuity pathway knowledge base as a reference set.

Results

GLI2 expression is associated with hormone responsiveness and androgen-independent progression. The expression levels of GLI2 mRNA in four prostate cancer cell lines were assessed by both Northern blot and quantitative RT-PCR (Fig. 1A and B). GLI2 mRNA expression was significantly higher in the androgen receptor—negative AI cell lines PC-3 and DU145 compared with androgen receptor—positive, androgen-sensitive cell lines LNCaP and C4-2 (P ≤ 0.05, respectively).

GLI2 staining in human prostate tissues was evaluated in the tissue microarrays (Fig. 1C and D; Supplementary Table S1). GLI2 expression, when present, was predominantly nuclear with weaker cytoplasmic expression in the epithelial cells. In the neoadjuvant hormone therapy (NHT) tissue array, GLI2 expression initially decreases in prostate cancers after androgen ablation when compared with untreated tumor (P ≤ 0.01); however, GLI2 expression increases again after prolonged hormone therapy (i.e., >6 months). Compared with benign prostate tissues (i.e., benign prostate hyperplasia group; P ≤ 0.05), GLI2 expression was significantly higher in untreated NHT and AI tumors. Moreover, because AI tumors exhibited higher intensity staining of GLI2 than other groups, targeted suppression would be most appropriate in this disease state.

Sequence-specific, dose-dependent inhibition of GLI2 by ASO in PC3 cells. GLI2 ASO significantly suppressed GLI2 expression in a dose-dependent and sequence-specific fashion at both mRNA and protein levels (Fig. 2). As shown in Fig. 2A and B, treatment of PC-3 cells with GLI2 ASO significantly reduced GLI2 mRNA levels by up to 89% in a dose-dependent manner.

![Figure 2](image-url)
with similar dose-dependent knockdown of GLI2 was observed at the protein level (Fig. 2C). The protein expression level of GLI1 was reduced in PC-3 cells treated with GLI2 ASO in a dose-dependent manner (Fig. 2C). Conversely, GLI2 expression was not modulated by GLI1 ASO in both mRNA and protein level (data not shown). These results are consistent with the previous report indicated that GLI1 was direct target of GLI2 (30).

![Fig. 3. Effect GLI2 ASO alone or in combination with paclitaxel on PC-3 cell growth in vitro.](#)

**Table 1.** Top 10 functional categories involved in GLI2 down-regulation

<table>
<thead>
<tr>
<th>Function</th>
<th>No. genes in class/no. genes measured</th>
<th>Significance*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell death and survival</td>
<td>53 /172</td>
<td>4.07e-05 to 2.96e-02</td>
</tr>
<tr>
<td>Reproductive system disease</td>
<td>23/172</td>
<td>4.07e-05 to 2.96e-02</td>
</tr>
<tr>
<td>Cancer</td>
<td>38/172</td>
<td>5.59e-05 to 2.96e-02</td>
</tr>
<tr>
<td>Cellular growth and proliferation</td>
<td>64/172</td>
<td>6.74e-05 to 3.04e-02</td>
</tr>
<tr>
<td>Cell cycle</td>
<td>22/172</td>
<td>4.49e-04 to 2.88e-02</td>
</tr>
<tr>
<td>DNA replication, recombination, and repair</td>
<td>13/172</td>
<td>4.49e-04 to 2.61e-02</td>
</tr>
<tr>
<td>Gene expression</td>
<td>44/172</td>
<td>7.45e-04 to 2.7e-02</td>
</tr>
<tr>
<td>Cellular development</td>
<td>10/172</td>
<td>7.73e-04 to 2.48e-02</td>
</tr>
<tr>
<td>Cell morphology</td>
<td>21/172</td>
<td>1.47e-03 to 2.91e-02</td>
</tr>
<tr>
<td>Cellular assembly and organization</td>
<td>15/172</td>
<td>1.47e-03 to 2.45e-02</td>
</tr>
</tbody>
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*Calculated and analyzed by Ingenuity pathway analysis. \( P < 0.05 \) is considered significant for association of genes.

\( ^{1} \) Number of genes found to be significantly changed (increased or decreased) as a function of GLI2 ASO in the corresponding function class.

\( ^{2} \) Number of genes in dataset file mapping for functional analysis.
GLI2 knockdown inhibits PC-3 cell growth and enhances chemosensitivity in vitro. The effect of GLI2 ASO monotherapy and its effects on paclitaxel chemosensitivity in vitro were investigated. PC-3 cells were treated for 2 days with 10 to 250 nmol/L GLI2 ASO or control ODN and growth rates were examined. Figure 3A shows significant reduction (***, \( P < 0.001 \)) of PC-3 cell growth 3 days after treatment with GLI2 ASO alone compared with control in a dose-dependent manner. To assess chemosensitivity after GLI2 ASO treatment, PC-3 cells were incubated with different concentrations of paclitaxel (0-10 nmol/L) and growth rates were examined daily for 2 or 4 days using crystal violet assay. Figure 3B reveals that 250 nmol/L ASO increases paclitaxel sensitivity compared with control plus paclitaxel treatment (*, \( P < 0.05 \); **, \( P < 0.01 \)). The combination index values of GLI2 ASO in combination with paclitaxel using CalcuSyn software at IC_{50} was 0.59, which indicates that the combination of GLI2 ASO and paclitaxel had a synergistic cytotoxic effect on PC-3 (31, 32).

GLI2 ASO treatment induces apoptosis in PC-3 cells in vitro. The induction of apoptosis by GLI2 ASO has been assessed by flow cytometry. The fraction of cells undergoing apoptosis (sub-G1-G0 fraction) was significantly higher after the treatment by flow cytometry. The fraction of cells undergoing apoptosis by GLI2 ASO or control ODN (20% versus 0.8%; ***, \( P < 0.001 \); Fig. 3C). Combination treatment using GLI2 ASO plus paclitaxel significantly increased sub-G1 fraction over that of paclitaxel plus control or ASO monotherapy (**, \( P < 0.01 \); 25.4% versus 11.7% and 25.4% versus 20.0%, respectively). The percentage of cells in G_{1} and G_{2}-M phase decreased after GLI2 ASO treatment, whereas an accumulation of cells at S phase was observed (data not shown). Furthermore, cells treated with GLI2 ASO expressed higher amounts of cleaved poly(ADP-ribose) polymerase products compared with cells treated with oligofectamine only and control (Fig. 3D).

Gene expression profile associated with GLI2 knockdown. Gene expression analysis was done with microarrays of 21,000 (70-mer) human oligonucleotides (Operon) to screen the genes involved in GLI2 ASO. In determining the potential downstream targets of GLI2, we identified 142 up-regulated genes and 180 down-regulated genes between PC-3 cells treated by GLI2 ASO or control ODN (Supplementary Tables S2 and S3). The identified genes were uploaded into the ingenuity pathway analysis knowledge database, which enables the discovery, visualization, and exploration of molecular interaction networks in gene expression data and was used to assess high-level function categories and networks (Table 1 and Supplementary Table S4). Supporting previous studies investigating GLI downstream targets (33, 34), cell death (apoptosis) and survival, cancer, cellular proliferation, and cell cycle were identified as high-level function categories in our ingenuity pathway analysis results. We focused on differentially expressed genes involved in apoptosis, cell survival, and cell cycle and did confirmatory Western blots to detect potential direct and indirect GLI2 targets.

Figure 4A illustrates several changes in levels of several genes linked with apoptosis and cell survival after GLI2 knockdown. Bcl-2 level decreased in PC-3 cell lysates treated with GLI2 ASO, which is consistent with previous reports that identified GLI2 interactions with the promoter of Bcl2 (30, 35). In addition Bcl-xL was significantly down-regulated in PC-3 cell treated by GLI2 ASO. The stress-induced cytoprotective chaperone, clusterin, was up-regulated in GLI2 ASO-treated cells, whereas levels of Mcl-1, an antiapoptotic Bcl-2 family member, was not altered among three groups.

Figure 4B showed the modulation of genes correlated with cell cycle by GLI2 knockdown. Genes regulating cell cycle enable cancer cells to evade antiproliferative signals (36), and hedgehog pathway and GLIs can interact with cell cycle machinery at various time points (34) by promoting D-type and E-type cyclins, thereby opposing P21-induced and P27-induced cell cycle arrest (37, 38). Compared with oligofectamine only and control ODN groups, levels of hyperphosphorylated Rb and cyclin D1 decreased in PC-3 cell lysates treated by GLI2 ASO, whereas protein levels of p27 were up-regulated. P21, whose mRNA level significantly increased in GLI2 ASO group in microarray data, did not express detectable protein levels using Western blot (data not shown), where its basal level has been reported to be very low in PC-3 cell (39). Interestingly, expression levels of PKC-\( \eta \) were significantly lower in PC-3 treated by GLI2 ASO in both mRNA and protein.
PKC-ε is expressed at higher levels in prostate cancer compared with benign prostate hyperplasia tissue (40) and reported to enhance the expression of G1 cyclin and p21 in MCF-7 adenocarcinoma cell line (41). These results indicate that GLI2 ASO modulates levels of several cell cycle genes, as well as genes correlated with G1-S phase to induce cell cycle arrest.

**GLI2 ASO treatment inhibits PC-3 tumor progression and enhances chemotherapy in vivo.** We next evaluated the effect of GLI2 ASO treatment on the growth of PC-3 tumors in vivo (Fig. 5). Male nude mice bearing PC-3 tumors (200 mm³) were randomly selected and treated with 15 mg/kg GLI2 ASO or control thrice a week by i.p. injection. From days 7 to 14 and 21 to 28, 0.5 mg/kg i.v. paclitaxel treatment on days 7 to 14 and 21 to 28. ASO or control ODN was continued at 15 mg/kg thrice a week. Points, mean tumor volume in each experimental group containing 10 mice; bars, SE. *, P ≤ 0.05; **, P ≤ 0.01; ***, P ≤ 0.001 (Student’s t test); ##, P ≤ 0.01; ###, P ≤ 0.001 (ANOVA repeated measurement).

C, total RNAs were extracted from the xenografts treated with GLI2 ASO or control ODN at 6 wk. GLI2 (left) and Bcl2 (right) mRNA expression levels were determined by quantitative RT-PCR. Columns, average values from five animals for each group; bars, SE. *, P ≤ 0.05 differ from control ODN by Student’s t test.

**Discussion**

Recent evidence suggests that the SHH-GLI pathway is activated in human prostate cancers (12, 13, 17). Expression of SHH, GLI1, and PTCH, which are known markers of SHH-GLI pathway activation, is increased in cancer compared with normal prostate epithelium (12). Furthermore, expression of SUFU, a negative regulator of the pathway, is inversely correlated with prostate cancer aggressiveness and tumor progression (13). Accordingly, activation of this pathway has been observed in prostate cancer metastases. Thus, the
SHH-GLI pathway seems to be associated with advanced prostate cancer and tumor progression (42). Interestingly, forced overexpression of SHH in LNCaP cells also activates its distal activator GLI and dramatically accelerates growth of tumor both in vitro and in vivo (11). Furthermore, disruption of the SHH-GLI pathway seems to inhibit prostate cancer cell growth. Upstream inhibition of the SHH pathway with either anti-SHH antibodies or SMO inhibitors, such as cyclopamine, can down-regulate GLI1 and inhibit proliferation and invasiveness of prostate cancer cell lines in vitro and in vivo (43). Accordingly, GLI transcription factors seem to be essential downstream effectors of the SHH pathway and may therefore be the most suitable targets to suppress the SHH pathway. In contrast to GLI1, which was originally identified as an oncogene (44), there is less evidence to implicate GLI2 in human malignancies. However, Sheng and colleagues showed that mice overexpressing GLI2 developed a variety of skin tumors, and GLI2 mRNA seemed to be up-regulated in many human skin tumor tissues (20). Our analysis of prostate cancer cell lines shows that GLI2 expression is higher in androgen receptor-negative androgen-insensitive cell lines, such as PC-3 and DU-145, compared with androgen receptor-positive, androgen-sensitive cell lines (Fig. 1). In human, GLI2 expression was significantly higher in untreated and AI prostate cancer tissues, decreasing temporally after androgen ablation therapy and increasing again after durations longer than 6 months of hormone therapy. These results suggest that GLI2 may correlate with development of castrate-resistant prostate cancer progression. Our functional studies showed GLI2 knockdown induced apoptosis and reduced tumor progression rates in the AI PC-3 cell line.

ASO are one strategy to specifically silence gene expression that specially hybridize with complementary mRNA region to inhibit target gene expression in a sequence-specific manner and important loss-of-function tools for in vitro and in vivo study (45). The GLI2 ASO (Isis 183652) used in this study incorporated a 2′-O-methoxymethyl gapmer modified backbone. These advanced chemistry ASO possess favorable physico-chemical, biochemical, and pharmacokinetic properties, with enhanced RNA affinity and reduced nonspecific immune stimulation (45). Most importantly, improved resistance against nuclease-mediated metabolism, resulting in significantly improved tissue half-life in vivo, enables a longer duration of action and a more intermittent dosing schedule. GLI2 levels were suppressed by 89% and associated with decreased cell growth in vitro and tumor growth in vivo. This study is the first to show the effect of GLI2 ASO therapy in prostate cancer cells either in vitro or in vivo.

We showed that GLI2 expression was increased in patients with hormone refractory prostate cancer. As well, our results showing the effects of GLI2 down-regulation imply that GLI2 overexpression may confer a growth advantage in this setting. Although further validation is warranted to investigate the mechanisms of GLI2 overexpression and the role of SHH in AI prostate cancer, the effect of GLI2 may be related to its relationship with other antiapoptotic proteins that have been shown to be increased in hormone refractory prostate cancer. Regl and colleagues suggested that the oncogenic activity of GLI2 may rely on at least three functions: stimulation of cell proliferation, repression of epidermal differentiation, and induction of prosurvival signals related with apoptosis (34, 35). They also identified several GLI2 targets, such as GLI1, Bcl2, and cell cycle genes (cyclin D1, E2F1, inactivation of Rb). The cell cycle inhibitors, P21 and p27, have also been reported as targets of GLI2 (10, 38). Our microarray data confirms that GLI2 knockdown induces changes in mRNA levels of diverse gene sets associated with survival, cancer, cellular proliferation, and cell cycle, indicating that apoptosis and cell cycle control may be the critical pathways modulated by GLI2. Furthermore, whereas we confirmed the downstream target genes shown by previous reports, such as Bcl2, GLI1, cyclin D1, and p27, we also detected Bcl-xl and PKC-η as new candidate targets modulated by GLI2.

Whereas a survival advantage of docetaxel-based chemotherapy has been shown in men with hormone refractory prostate cancer, these improved effects were modest, underscoring the need for additional therapeutic reagent (3, 4). Previous studies reported that Bcl2 and Bcl-xl confers resistance to taxane chemotherapy in several prostate cancer models (26, 29, 46–48). In this current study, GLI2 knockdown was associated with decreased levels of Bcl2, increased apoptosis, and paclitaxel chemosensitization both in vitro and in vivo. Our current hypothesis is that synergy may be achieved through the possible modulation of Bcl2-related antiapoptotic that seems to also be down-regulated after ASO transfection. Furthermore, increased clusterin levels after GLI2 knockdown is consistent with clusterin’s role as a stress-induced cytoprotective chaperone that inhibits apoptosis after a diverse array of anticancer treatments. OGX-011, a second generation ASO inhibitor of clusterin, is currently in clinical trials of prostate, breast, and lung cancer, and future preclinical studies examining effects of combined suppression GLI2 and clusterin are under way (47, 50). The GLI2 ASO used in this study has a potential to be developed as a monotherapy or in combined multimodality regimens with chemotherapy.

In summary, the present study suggests that GLI2 is an attractive therapeutic target in prostate cancer by affecting the expression of many genes that regulate apoptosis and cell cycle. We show that GLI2 knockdown induces apoptosis, inhibits cancer growth, and chemosensitizes cells to chemotherapy in vitro and in vivo. These results provide preclinical proof of principle for the use of this novel therapeutic in castrate-resistant prostate cancer.

Disclosure of Potential Conflicts of Interest

Y. Kim owns stock in ISIS Pharmaceuticals.

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GLI2 Knockdown Using an Antisense Oligonucleotide Induces Apoptosis and Chemosensitizes Cells to Paclitaxel in Androgen-Independent Prostate Cancer

Shintaro Narita, Alan So, Susan Ettinger, et al.


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