Arsenic attenuates GLI signaling, increasing or decreasing its transcriptional program in a context dependent manner


Molecular Oncology Program (B.L., C.G., E.W., J.L., K.J., Z.W., D.L.F., D.M.N., A.J.C., D.J.R.), Department of Surgery, Miller School of Medicine, University of Miami, Miami, FL 33136, USA; General Surgery Center of PLA (B.T.), Southwest Hospital, the Third Military Medical University, Chongqing, 400038, China; Sheila and David Fuente Graduate Program in Cancer Biology (J.L.), Miller School of Medicine, University of Miami, Miami, FL 33136, USA; Program in Experimental and Molecular Medicine (D.L.F.), Department of Pharmacology and Toxicology, Geisel School of Medicine at Dartmouth, Hanover, NH 03755; Sylvester Comprehensive Cancer Center (D.M.N., P.R., P.R.S., B.A., A.J.C., D.J.R.), Miller School of Medicine, University of Miami, Miami, FL 33136, USA; Department of Dermatology Volker Hall (M.A.), The University of Alabama at Birmingham, Birmingham, AL 35294, USA; Department of Genetic Medicine (B.W.), Weill Medical College of Cornell University, 1300 York Ave., New York, NY 10065, USA; Department of Public Health Sciences (L. W.), Division of Biostatistics, Miller School of Medicine, University of Miami, Miami, FL 33136, USA; Department of Medicine (R.R.S., P.R., B.A.), Miller School of Medicine, University of Miami, Miami, FL 33136, USA; Division of Geriatric Medicine and Palliative Care (P.R.), Miller School of Medicine, University of Miami, Miami, FL 33136; Department of Biochemistry and Molecular Biology (A.J.C., D.J.R.), Miller School of Medicine, University of Miami, Miami, FL 33136, USA;
Running title: Arsenic trioxide plays dual roles in GLI-dependent signaling

*To whom correspondence should be addressed: Dr. David J. Robbins, Molecular Oncology Program, Department of Surgery, Miller School of Medicine, University of Miami, Miami, FL 33136, USA.
Tel: (305)-243-5717; Fax: (305)-243-1421; Email: drobbins@med.miami.edu

Abbreviations:
ATO, Arsenic Trioxide; HH, Hedgehog signaling; GLI-FL, GLI full length; GLI-A, GLI activator; GLI-R, GLI repressor; FDA, Food and Drug Administration; PTCH1, Patched1; SMO, Smoothened; NIH, National Institutes of Health; ATCC, American Type Culture Collection; RNA, ribonucleic acid; qRT-PCR, quantitative reverse transcription polymerase chain reaction; PBS, phosphate-buffered saline; WBC, white blood cell; WT, wild type; SUFU, Suppressor of fused. SAG, Smoothened agonist; MEF, mouse embryonic fibroblasts.
Abstract

The metalloid arsenic is a worldwide environmental toxicant, exposure to which is associated with many adverse outcomes. Arsenic is also an effective therapeutic agent in certain disease settings. Recently, arsenic was shown to regulate the activity of the Hedgehog (HH) signal transduction pathway, and this regulation of HH signaling was proposed to be responsible for a subset of arsenic's biological effects. Surprisingly, these separate reports proposed contradictory activities for arsenic, as either an agonist or antagonist of HH signaling. Here we provide in vitro and in vivo evidence that arsenic acts as a modulator of the activity of the HH effector protein GLI, activating or inhibiting GLI activity in a context-dependent manner. This arsenic induced modulation of HH signaling is observed in: 1) cultured cells, 2) colorectal cancer patients who have received arsenic based therapy, and 3) a mouse colorectal cancer xenograft model. Our results show that arsenic activates GLI signaling when the intrinsic GLI activity is low, but inhibits signaling in the presence of high-level GLI activity. Further, we show that this modulation occurs downstream of primary cilia, evidenced by experiments in SUFU−/− cells. Combining our findings with previous reports, we present an inclusive model in which arsenic plays dual roles in GLI signaling modulation: when GLIs are primarily in their repressor form, arsenic antagonizes their repression capacity, leading to low-level GLI activation, but when GLIs are primarily in their activator form, arsenic attenuates their activity.
Introduction

Arsenic is a widespread environmental toxicant that poses a significant threat to human health (Rahman et al., 2009). Chronic exposure to arsenic has been linked to increased risk of a variety of adverse human health outcomes including cancer and developmental defects (IARC, 2004; NRC, 1999). In contrast, formulations of arsenic have previously exhibited efficacy against a variety of human diseases, such as syphilis, agues, malaria and leukemia (Au, 2011). Arsenic trioxide (ATO) is currently FDA-approved for the treatment of acute promyelocytic leukemia (Mi, 2011). Despite the long history of human exposure to and usage of arsenic, the exact mechanisms by which arsenic exerts these biological effects remain obscure.

The Hedgehog (HH) signaling pathway is one of the primary signal transduction pathways that govern the rapid growth and patterning of vertebrate embryos and regulate adult tissue homeostasis (Ingham and McMahon, 2001). Consistent with the important role that HH signaling plays during development and tissue homeostasis, deregulation of HH signaling leads to an array of developmental disorders and many types of cancer (Altaba, 2006; Barakat et al., 2010; Jiang and Hui, 2008). The HH family of ligands bind to their primary cellular receptor Patched1 (PTCH1) to initiate signaling (Robbins and Hebrok, 2007). Upon HH binding, PTCH1 releases its inhibitory effect on Smoothened (SMO), which subsequently triggers a series of cellular events that regulate the stability, proteolytic processing, and activity of the GLI family of transcription factors (GLI1-3) (Robbins and Hebrok, 2007; Ruiz i Altaba et al., 2007). In the absence of HH, the full-length forms of GLI2 and GLI3 (GLI-FL) are processed into truncated repressor forms (GLI-R). In the presence of HH, this partial proteolysis is attenuated and instead GLI-FL is converted into its activated form (GLI-A). GLI trafficking
through primary cilia is required for the conversion of GLI-FL to either its GLI-R or GLI-A forms (Robbins et al., 2012).

We previously suggested that HH pathway activation underlies the etiology of some arsenic-induced diseases (Fei et al., 2010). We showed that both short-term and chronic arsenic exposure activates HH signaling in primary cells, established cell lines, and certain tissues of exposed mice. We further showed that arsenic exposure levels are positively associated with HH pathway activity in human bladder cancer, a malignancy previously linked to chronic arsenic exposure (Fei et al., 2010). More recently, other reports have shown that arsenic inhibits HH signaling initiated by either HH treatment or high GLI levels, and concluded that arsenic is a functional inhibitor of HH/GLI signaling (Beauchamp et al., 2011; Han et al., 2013; Kim et al., 2013; Kim et al., 2010; Nakamura et al., 2013; Yang et al., 2013; You et al., 2013).

Here, we provide in vitro and in vivo evidence suggesting that arsenic can both activate and inhibit GLI signaling, with the direction of GLI modulation dictated primarily by the starting levels of intrinsic GLI activity. Further, we provide a unifying model that explains how arsenic acts mechanistically to modulate GLI activity.

**Materials and Methods**

**Cell culture, reagents, and assays**

NIH-3T3 cells (ATCC), and the colorectal cancer (CRC) cell lines SW480, DLD-1 and HCT116 (gifts from Dr. Ethan Lee, Vanderbilt University), were cultured as previously described (Li et al., 2014b). ATO (Trisenox, Cephalon) was used at the indicated concentrations, or combined with recombinant HH (rHH C25II, R&D Systems), on cultured cells. RNA extraction, reverse
transcription, quantitative PCR (qRT-PCR), luciferase reporter assays and plasmids expressing MYC tagged human GLI1 or GLI2 were previously described (Fei et al., 2010; Li et al., 2014a). MYC tagged GLI3-R (expressing truncated GLI3 which lacks the GLI transcriptional activation domain) was a gift from Dr. Ariel Ruiz i Altaba (University of Geneva Medical School).

**Mouse experiments**

Animal experiments were conducted in accordance with protocols approved by the Institutional Animal Care and Use Committee at the University of Miami. HCT116 cells (2×10^6) were injected subcutaneously onto the left flank area of 4- to 5-week-old female nude mice (Crl:NU-Foxn1^nu^). When the xenograft tumor reached a size of approximately 200 mm^3^, the mice were randomized into two groups and treated by intraperitoneal injection with vehicle (PBS) or 10 mg/kg of ATO. After two days of treatment, the tumors and kidneys were harvested and RNA extracted for qRT-PCR analysis.

**Patient samples**

Details of the patient selection and treatment plan were previously described (Clinical Trial Number: NCT00449137) (Ardalan et al., 2010). Briefly, the enrolled patients harbored CRC at advanced metastatic stages, and were refractory to at least two standard chemotherapy regimens including 5-fluorouracil. These patients were treated with ATO (0.15 - 0.20 mg/kg) for 2 to 10 treatment cycles, along with intermittent doses of 5-fluorouracil (Ardalan et al., 2010). Biopsies of CRC liver metastases and white blood cells (WBCs) were taken within one week prior to the start of ATO treatment, on day 23 of the first cycle, and in one case (ID#1) after
completion of the last treatment cycle. All fine needle biopsies of CRC liver metastases were confirmed as malignant cells before further processing. Biopsies were stored in RNA later (Ambion) before total RNA isolation. White blood cells were isolated as previously described (Subbarayan et al., 2002). Total RNA was isolated using Trizol (Invitrogen) and purified using RNAeasy kits (Invitrogen), followed by gene expression analysis using qRT-PCR.

Statistical analysis

Experiments were independently performed multiple times. Statistical significance was determined by Student's t test unless otherwise indicated. In all analyses, a p-value < 0.05 was considered statistically significant.

Results

As previous reports of arsenic's effect on GLI/HH signaling have been contradictory (Beauchamp et al., 2011; Fei et al., 2010; Kim et al., 2013; Kim et al., 2010), we reexamined this question using experimental systems capable of showing both increases and decreases in GLI activity. As ultimately our interest is in the effects of arsenic on human pathologies, we first examined the effects of ATO on GLI target genes in patient specimens obtained as part of an ATO clinical trial for colorectal cancer (Ardalan et al., 2010). Needle biopsies of CRC liver metastases, along with cognate non-tumorigenic WBCs, were taken from these patients, before, during, and in one patient, after termination of ATO treatment. The expression of GLI target genes was subsequently examined in these primary tissue samples. For patient ID#1, from whom we had all three tissue samples, ATO attenuated GLI target gene expression, and
did so in a manner that was reversible upon ATO withdrawal (Fig 1A). In contrast, ATO treatment activated GLI target genes in WBCs from this same patient (Fig 1B). Notably, these effects on GLI signaling by ATO were also reversible upon ATO withdrawal (Fig 1A and B). When we extended this analysis to the five patients in our cohort for which we had such tissue samples (Fig 1C), the expression of GLI/HH biomarkers was downregulated in the majority of ATO treated patient tumor samples (4 out of 5 cases for *GLI1* and 2 out of 5 cases for *PTCH1*), while *PTCH1* was upregulated in WBCs in 3 out of 5 cases. Although the small numbers of patient samples examined precludes a generalization of these results, we were able to observe distinct modulation of GLI/HH signaling in tumors and WBCs within the same patient. This differential modulation of HH signaling in vivo occurred in response to therapeutically relevant doses of arsenic (Shen et al., 1997). Interestingly, *GLI1* expression levels were higher in the tumor samples than in the matching WBC samples (Supplemental Figure 1), consistent with previous reports showing higher levels of GLI activity in CRC metastases (Varnat et al., 2009).

As we were unable to make statistically relevant conclusions regarding GLI modulation using our small cohort of arsenic treated CRC patients, we developed a CRC xenograft mouse model and examined the effect of ATO on GLI activity in the CRC and mouse tissue. HCT116 cells were used in this assay, because this cell line, along with two other CRC cell lines, requires high levels of GLI activity (Mazumdar et al., 2011a; Mazumdar et al., 2011b; Varnat et al., 2009) for viability, and is responsive to ATO in vitro (Fig 2A and B). HCT116 cells were implanted subcutaneously onto the flanks of immunocompromised nude mice. Once the tumors reached a size of approximately 200 mm$^3$, mice were randomized to two treatment
groups and were given daily doses of ATO (10 mg/kg) or vehicle for two days. Tumor tissues or mouse kidneys, which we have previously shown to respond to arsenic by increasing GLI signaling (Fei et al., 2010), were harvested from these mice, and the expression of \textit{GLI1} and \textit{PTCH1} determined by quantitative RT-PCR. ATO treatment dramatically decreased the expression of GLI target genes in the CRC xenograft tumors (Fig 2C). However, ATO also increased basal GLI activity in the kidneys of these same mice (Fig 2D). Combined, these data suggest that ATO treatment can both decrease and increase GLI activity in the same experimental system, albeit in a tissue dependent manner.

We hypothesized that ATO might modulate GLI activity in a manner dependent on the basal levels of GLI activity, which would be dictated by the GLI-R/GLI-A ratio (Ruiz i Altaba et al., 2007). To test this hypothesis, we examined the effects of ATO on GLI activity in NIH-3T3 fibroblasts, whose GLI signaling level can be modulated by HH ligands or Smoothened agonists (Fei et al., 2010; Kim et al., 2010). We treated NIH-3T3 cells with increasing doses of ATO in the absence of HH ligand, and noted that ATO elevated the expression of GLI biomarkers (Fig 3A, left panel), consistent with our previous report (Fei et al., 2010). However, when NIH-3T3 cells were treated with ATO in the presence of HH, which activates GLI-A but attenuates GLI-R activity, \textit{GLI1} and \textit{PTCH1} levels were attenuated in a dose-dependent manner (Fig 3A, right panel). These results support the hypothesis that ATO can both activate and attenuate GLI signaling depending on its basal state, and in this case does so in a single cell type. An alternative possibility is that cells with different GLI signaling levels may have differential rates of arsenic transport or methylation, and thus production of arsenic metabolites, which may have distinct effects on GLI signaling. However, we tested the expression of the
major arsenite transporters, \textit{AQP7} and \textit{AQP9}, and arsenite methyltransferase, \textit{AS3MT}, in WT mouse embryonic fibroblasts (MEFs) and didn’t find it to be significantly different (Supplemental Figure 2) under conditions of low-level versus high-level GLI signaling.

We speculated that a unified mechanism to explain the seemingly conflicting effects of ATO on GLI activity might exist. One plausible hypothesis is that ATO targets the primary cilium, which is suggested to play dual roles in HH signaling (Han et al., 2009). Furthermore, one previous report indicated that ATO decreased the trafficking of GLI2 through primary cilia (Kim et al., 2010). We therefore tested the ability of ATO to attenuate HH signaling in MEFs lacking \textit{SUFU}, a pivotal negative regulator of HH signaling, whose increased levels of GLI activity are activated independently of primary cilia (Zhang et al., 2009). ATO attenuated the constitutive GLI activity of \textit{SUFU}^/- MEFS, but activated GLI activity in WT MEFs (Fig 3B). Therefore, ATO can attenuate GLI activity downstream of SMO, independently of primary cilia, inconsistent with the hypothesis that ATO attenuates GLI-A by affecting its trafficking through the primary cilium.

We previously reported that the levels of GLI3-R were decreased in cells treated chronically with ATO (Fei et al., 2010), suggesting that the agonist effect of ATO may come from the degradation of GLI3-R protein. However, we found here that the agonist effect of ATO could be observed as early as 6 hours (Fig 3C), prior to any detectable GLI3-R degradation (Fig 3D & E). To further explore the relationship between ATO exposure and GLI activity, we took advantage of immortalized \textit{GLI2}^/-;\textit{GLI3}^/- MEFs that lack the major repressor and activator GLI forms and consequently do not respond to HH (Lipinski et al., 2008). We transfected plasmids
expressing GLI1 or GLI2, the major GLI-A forms, into these cells to activate GLI signaling. This exogenous GLI activity was subsequently attenuated by ATO (Fig 4A). We next investigated the mechanism of GLI activation by ATO using WT and GLI2\textsuperscript{-/-};GLI3\textsuperscript{-/-} MEFs, validating that ATO’s activation of GLI signaling requires GLI2 and/or GLI3 (Fig 4B) (Fei et al., 2010). To further assess the impact of ATO on the dominant GLI-R, GLI3, we utilized GLI3\textsuperscript{-/-} MEFs. In these cells, the loss of GLI3-R activity results in a net increase in basal GLI activity (Lipinski et al., 2008), driven by unrepressed, basal GLI2 activity. Transfection of a plasmid expressing GLI3-R into these cells repressed PTCH1 expression to levels comparable with those of WT MEFs. ATO was capable of reversing this GLI3-R activity (Fig 4C), and did so in a manner that did not decrease GLI3-R protein levels (Fig 4D). This result is consistent with ATO acting directly to repress GLI3-R activity, with degradation of GLI3-R being a secondary effect of ATO subsequent to attenuation of its activity. Taken together, these results suggest that ATO is capable of attenuating both GLI-A and GLI-R activities, depending on which one is dominant in the cells.

Discussion

The mechanism and direction of modulation of GLI/HH signaling by arsenic has been controversial. We have previously shown, and also demonstrate here, that short and long-term exposure to arsenic activates GLI signaling. However, a number of other groups have argued that arsenic attenuates GLI signaling rather than activate it (Beauchamp et al., 2011; Fei et al., 2010; Han et al., 2013; Kim et al., 2013; Kim et al., 2010; Nakamura et al., 2013; Yang et al., 2013; You et al., 2013). We now show, in agreement with these previous groups, that arsenic can also antagonize GLI/HH signaling. Further, we advance this work by showing that GLI
activity can be activated or attenuated by arsenic in the same experimental systems, \textit{in vitro} and \textit{in vivo}. The disparate modulation of GLI activity we observe in these identical experimental systems is consistent with the modulation of GLI activity being context dependent, rather than resulting from experimental vagaries that can sometimes occur between different laboratories. Our NIH-3T3 data show that altering arsenic concentrations can both increase and decrease Gli signaling in a dose dependent manner, depending on the presence or absence of HH. It is therefore unlikely that differential expression levels of arsenic transporters or arsenite methyltransferase is the primary mechanism responsible for the dual regulatory capacity of GLI/HH signaling by arsenic, at least in a single cell line. However, we cannot rule out that, in more complicated systems such as human cancer patient samples, organic arsenic metabolites may be involved in the regulation of GLI activity by arsenic, although inorganic arsenic has been reported to directly bind to GLI proteins (Beauchamp et al., 2011; Han et al., 2013), suggesting that inorganic arsenic is the main active arsenic species that modulates GLI activity.

Our results suggest that ATO acts as an unbiased GLI inhibitor, attenuating both its transcriptional activation and repressor activity. Based on results from a number of labs, including ours, we suggest that ATO attenuates GLI-A and GLI-R activity by binding directly to their zinc finger domains to disrupt their transcriptional regulatory functions. These effects occur downstream of primary cilia, on GLI proteins bound to the regulatory elements of their target genes. Alternatively, as arsenic has been implicated in global epigenetic modulation (Ren et al., 2011), ATO might regulate the activity of GLI target genes via epigenetic mechanisms. We do not favor this latter mechanism, as ATO is also capable of modulating the
effects of exogenous GLI-A and GLI-R on a GLI-driven luciferase reporter gene, which should not be regulated by epigenetic mechanisms. We present here a unifying model to explain these cell context specific responses to arsenic (Fig 5): In cellular contexts with low GLI activity, GLI3-R plays a dominant role in repressing target gene transcription (Litingtung and Chiang, 2000; Pan et al., 2006) and ATO consequently binds to GLI3-R to attenuate this repression, which leads to increased GLI signaling. In cellular contexts harboring high GLI-A activity, ATO binds to GLI-A to attenuate GLI activity.

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Author contributions
Conducted experiments: B.L.; C.G.; B.T.; E.W.; J.L.; K.J.; D.L.F.; P.R.S.
Contributed new reagents or analytical tools: C.G.; B.W.
Performed data analysis: B.L.; C.G.; L.W.; A.J.C.; D.J.R.
Wrote or contributed to the writing of the manuscript: B.L.; E.W.; P.R.S.; A.J.C.; D.J.R.
References


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**Footnotes**

B.L., C.G. and B.T. contributed equally to this work.

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Figure legends

Figure 1. ATO modulates GLI activity in CRC patients.

RNA was extracted from the tumor (A) or associated white blood cells (B) of a colorectal cancer patient who received ATO in the clinic. The expression of GLI target genes relative to that of GAPDH was determined by qRT-PCR. Error bars indicate standard error of triplicate experiments using one sample. Similar unnormalized data is shown in supplemental Figure 1. (C) Summary of GLI target gene expression in the tumors and associated WBCs of a group of CRC patients who received ATO treatment.

Figure 2. ATO modulates GLI signaling in a CRC xenograft mouse model.

CRC cells (DLD-1, SW480 and HCT116) were treated with the indicated concentrations of ATO for 48 hours, and GLI1 (A) or PTCH1 (B) expression relative to that of GAPDH was determined by qRT-PCR. (C & D) 2x10^6 HCT116 cells were subcutaneously implanted into nude mice. When the tumor size reached ~200 mm^3, 10 mg/kg ATO or vehicle control were injected for two consecutive days. Tumor and kidney samples were then harvested for RNA extraction and the expression of GLI1 and PTCH1 relative to that of GAPDH was determined by qRT-PCR. Error bars represent S.D. (n = 10) and a * indicates p < 0.05 calculated using a Wilcoxon signed rank test. Normalized data is shown here because of the inherent difficulty in comparing mouse expression levels to human expression levels.

Figure 3. ATO exerts context dependent modulation of GLI signaling and does so independently of primary cilia.
(A) NIH-3T3 cells were treated with the indicated dose of ATO with or without 1 µg/ml recombinant HH for 48 hours, and the expression of the indicated target genes relative to that of GAPDH was determined. (B) WT or SUFU<sup>−/−</sup> MEFs were treated with the indicated dose of ATO for 48 hours. The expression of GLI1 relative to that of GAPDH was then determined by qRT-PCR. (C) NIH-3T3 cells were treated with vehicle, 3 µM ATO or 100 nM SAG for 6 hours, and PTCH1 expression relative to that of GAPDH was determined by qRT-PCR. (D & E) NIH-3T3 cells were treated with either 100 nM SAG or 3 µM ATO for 6 hours, and full-length GLI3 and GLI3-R levels were determined by immunoblotting. A representative immunoblot (D) and a quantification of multiple independent immunoblots (E) are shown. The top left panel (vehicle treated NIH-3T3 cells or WT MEFs) in each figure was set as 1 for direct comparison in all figures. Error bars represent S.E.M (n = 3) and a * indicates p < 0.05.

Figure 4. ATO attenuates the activity of the activator and repressor forms of GLI proteins.

(A) GLI2<sup>−/−;</sup>GLI3<sup>−/−</sup> MEFs were transfected with a plasmid expressing a GLI luciferase reporter gene with or without GLI1 or GLI2 expression plasmids, and then treated with the indicated doses of ATO. Luciferase activity relative to total protein level was measured after 48 hours of treatment. The activity of a vehicle treated and mock-transfected sample (left panel) was set as 1 for comparison. Error bars represent S.E.M. (n = 3) and a * indicates p < 0.05. (B) GLI2<sup>−/−;</sup>GLI3<sup>−/−</sup> or WT MEFs were transfected with a plasmid expressing a GLI luciferase reporter gene, and then treated with the indicated doses of ATO. Luciferase activity relative to total protein level was then determined. The activity of a vehicle treated sample (left panel, WT MEFs) was set as 1 for comparison. Error bars represent S.E.M. (n = 3) and a * indicates p < 0.05. (C)
GLI3−/− MEFs were transfected with increasing amounts of a GLI3-R expressing plasmid and then treated with vehicle or 3 µM ATO. PTCH1 expression relative to that of GAPDH was measured as a readout of GLI activity. PTCH1 expression relative to that of GAPDH in WT MEFs was also determined as a control. Expression in a vehicle treated mock transfected sample (left panel) was set as 1 for comparison. Error bars represent S.E.M. (n = 3) and a * indicates p < 0.05. (D) GLI3−/− MEFs were transfected with the indicated amounts of GLI3-R expression plasmids and then treated with vehicle or 3 µM ATO. GLI3-R (a long and short exposure) or GAPDH levels were revealed by immunoblotting. A representative result is shown.

Figure 5. A schematic model of how ATO modulates GLI/HH signaling in different contexts.

Arsenic trioxide suppresses the activity of GLI proteins in both their activator and repressor forms. In cells with a low level of HH signaling, arsenic inhibits the repressor forms of GLI and thus results in a net increase in basal HH signaling (agonist mode). In cells with high levels of HH signaling, arsenic serves as an inhibitor of the activator forms of GLI (antagonist mode) and therefore decreases HH signaling.
Figure 1

A. Tumor sample

- Pre-treatment
- During treatment
- Post treatment

Gene expression

GLI1

PTCH1

B. WBC sample

- Pre-treatment
- During treatment
- Post treatment

Gene expression

GLI1

PTCH1

C. Patient ID | GLI1 levels | PTCH1 levels
--- | --- | ---
#1 | DOWN | DOWN | UP | UP
#2 | UP | ND | UP | ND
#3 | DOWN | DOWN | NC | UP
#4 | DOWN | ND | DOWN | UP
#5 | DOWN | DOWN | NC | DOWN
Figure 2

A

GLI1 expression

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PTCH1 expression

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C

GLI1 expression

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Tumor

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Kidney

PTCH1 expression

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Figure 3

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<th>SUFU/- MEF</th>
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<td><img src="image21" alt="GLI1 expression" /></td>
<td><img src="image22" alt="GLI1 expression" /></td>
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C

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<th>PTCH1 expression</th>
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<tr>
<td>ATO</td>
<td><img src="image24" alt="PTCH1 expression" /></td>
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<td>SAG</td>
<td><img src="image25" alt="PTCH1 expression" /></td>
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D

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E

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<td>ATO</td>
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<td>SAG</td>
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Figure 5

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