Role of GLI2 in growth of human osteosarcoma

Objective: Hedgehog (Hh) pathway has been implicated in different aspects of animal development, acting through several components, including the transmembrane proteins PATCHED1 (PTCH1) and SMOOTHEENED (SMO), to activate the GLI zinc-finger transcription factors. Hh pathway is critical for many processes during embryonic and postnatal development. Recent studies have demonstrated constitutive activation of Hh pathway in various types of malignancies. However, it remains unclear how Hh pathway is involved in the pathogenesis of osteosarcoma. To explore the involvement of aberrant Hh pathway in the pathogenesis of osteosarcoma, we investigated the expression and activation of Hh pathway in osteosarcoma and examined the effect of GLI2 inhibition by a specific inhibitor of GLI2 or GLI2 RNAi. In addition, we examined the effect of forced expression of constitutive-active GLI2 by expression vector.

Methods: To evaluate the expression of genes of Hh pathway, we performed real-time PCR using osteosarcoma cell lines and osteosarcoma biopsy specimens from patients. We evaluate the effect of GLI2 inhibition in vitro using MTT assay, colony formation assay, and western blot. We evaluate the effect of GLI2 inhibition in vivo by xenografts models. We transfected the constitutive-active GLI2 expression vector, and cell growth.

Results: Over-expression of Hh-GLI pathway molecules in osteosarcoma. To explore the role of Hedgehog pathway in osteosarcoma, we examined the expression of Hedgehog in osteosarcoma cell lines. Real-time PCR revealed that 4 of 5 human osteosarcoma cell lines increased Sonic Hedgehog. In addition, 5 of 5 osteosarcoma cell lines increased Desert Hedgehog. To further examine Hedgehog pathway molecules expression, we performed real-time PCR for Hedgehog receptors and Hedgehog target genes. 5 of 5 human osteosarcoma cell lines increased PTCH1. Four of 5 human osteosarcoma cell lines increased SMO. Five of 5 human osteosarcoma cell lines increased GLI1. Five of 5 human osteosarcoma cell lines increased GLI2. We next examined SMO expression in osteosarcoma patient’ biopsy specimens. Real-time PCR revealed that 9 of 9 human biopsy specimens of osteosarcoma increased SMO. In addition, expression of PTCH1 was increased in 8 of 9 patients’ biopsy specimens. Nine of 9 human biopsy specimens increased GLI2. Of most importance was the finding that markers of active Hedgehog signaling, GLI2 and PTCH1 were consistently up-regulated. Of most importance was the finding that markers of active Hedgehog signaling, GLI2 and PTCH1 were consistently up-regulated. Our findings suggest that Hedgehog signaling is active in osteosarcomas.

Inhibition of GLI2 prevents osteosarcoma growth in vitro

To examine the effect of GLI2 suppression, we examined the effect of GLI2 siRNA. MTT assay revealed that knock-down of GLI2 prevented osteosarcoma growth (Fig. 1). Colony formation assay revealed GLI2 siRNA reduced colony formation in soft agar (Fig. 1). These findings show that suppression of GLI2 prevents osteosarcoma growth in vitro.

Hh-GLI signaling regulates cell cycle of osteosarcoma

We examined cell cycle characteristics by flow cytometry. Of control shRNA transfected-143B cells, 72.5 % of cells were in G1 phase, while 81.6% of cells were in G1 phase following GLI2 shRNA transfection. Real-time PCR revealed that GLI2 shRNA prevented the transcription of accelerators of the cell cycle including cyclin D1 and SKP2. Western blot showed that GLI2 shRNA reduced the levels of expression of cyclin D1, pRb, and SKP2 proteins. We next examined the expression of p21<sup>WAF</sup>, and found that p21<sup>WAF</sup> protein was up-regulated by GLI2 shRNA. These findings suggest that inhibition of GLI2 inhibited osteosarcoma growth via cell cycle regulation.

Forced expression of constitutive active GLI2 promote mesenchymal stem cell growth by cell cycle regulation

We next examined the effect of forced expression of GLI2. MTT assay revealed that forced expression of constitutive active GLI2 promoted YKNK mesenchymal stem cell growth. Colony formation assay showed that forced expression of constitutive active GLI2 increased colony formation in soft agar. Cell cycle analysis showed that forced expression of constitutive active GLI2 decreased G1 phase cells and increased S-G2-M phase cells.

Knock down of GLI2 prevents osteosarcoma growth in vivo

To confirm the effect of GLI2 suppression, we used GANT61, a pharmacological agent known to effectively block Hh-GLI signaling by inhibiting GLI2 activation. MTT assay showed that GANT61 slowed the growth of 143B. These findings show that suppression of GLI2 prevents osteosarcoma growth in vitro. Nude mice were inoculated with control shRNA or GLI2 shRNA transfected 143B osteosarcoma cells intradermally. Results demonstrated significant inhibition of tumor growth GLI2 shRNA versus control shRNA (Fig. 2). Kaplan-Meier analysis showed that GLI2 shRNA conferred a significant survival benefit (Fig. 2). These findings suggest that inhibition of GLI2 prevents osteosarcoma growth in vivo.

Conclusions: We previously reported that inhibition of SMO prevents osteosarcoma growth in vitro and in vivo (Hirotsu M.et al. Molecular Cancer 2010). Our new findings confirm that the Hh pathway is functionally activated in osteosarcoma. Our findings suggest that inactivation of GLI2 may be an attractive target for the treatment of patients with osteosarcoma.

Figure 1

![Figure 1](attachment:image1.png)

Figure 2

![Figure 2](attachment:image2.png)