INDIAN HEDGEHOG SIGNALING IN OSTEOSARCOMA: CO-EXPRESSION OF IHH, PTCH1, AND GLI1

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INTRODUCTION:
Osteosarcoma is a primary bone cancer, which is often associated with rapid bone growth so its highest incidence is in teenagers. It is a rare disease, which affects only 2 per million people per year. Before the modern era of therapy combining surgery, and chemotherapy, less than 10% of patients were cured. The overall survival for patients has increased to 50-70%. Further improvements to reduce the risk of metastatic disease, which affects 30-50% of all patients, is still under investigation.

The Hedgehog (Hh) signaling pathway, which was first identified in Drosophila melanogaster, is crucial in the developmental processes of numerous tissues and organs especially during embryonic development by influencing the transcription of many target genes. Dysregulation of Hh signaling was demonstrated in cancers of the skin, cerebellum muscle, digestive tract, pancreas, breast, and prostate. Indian Hedgehog (IHH) signals through the transmembrane proteins patched (PTCH) and smoothened (SMO) to activate GLI mediated transcription, including GLI1 and PTCH1. This exerts a feedback mechanism to regulate further IHH signaling.

We previously demonstrated that chondrosarcoma expressed high levels of the Hedgehog target genes, PTCH1 and GLI1. Moreover, reduced proliferation of chondrosarcoma primary tissue specimens was observed when the IHH pathway was blocked by cyclopamine, a steroid alkaloid that inhibits Hedgehog signaling through direct interaction with SMO (1).

The purpose of the study was to characterize the expression of IHH, PTCH1, and GLI1 in 43 primary tumor specimens to determine the role of IHH signaling in osteosarcoma.

METHODS:
Tumor specimens and osteosarcoma cell lines were both used in this study. Fresh frozen tumor specimens were obtained from 43 patients with high grade osteosarcoma of the extremity, including 11 who presented with metastases at diagnosis (MetDx), and 32 who had no systemic disease at presentation (NoMetDx). Six osteosarcoma cell lines obtained from ATCC were U-2 OS, Saos-2, MG-63, MNNG/HOS, HOS, and KHOS/NP. RNA was extracted from the specimens and cell lines. Real time RT-PCR (Gene-specific TaqMan Assay-on-Demand) was used to assess the expression level of IHH, GLI1, PTCH1. The internal control gene for IHH in each reaction was Asparagine synthetase (AS), for GLI1 and PTCH1 were Glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The absolute standard curve method was used to determine the copy number of the transcript of interest. cDNA from colon cell lines and a pool of 11 tumor cell lines were used as reference standards.

For the purposes of statistical analysis, the expression data for IHH, GLI1, and PTCH1 were log transformed in order to achieve approximate normality of the error distribution. Satterthwaite’s test for unequal variance was used for tests comparing outcome groups. Non-parametric tests (Wilcoxon for binary and Jonkheere-Terpstra for continuous variables) were used to assess the association between expression levels and selected clinical and demographic variables.

RESULTS:
We found that the expression level IHH, GLI1, and PTCH1 varies among tumor samples. Among the 6 osteosarcoma cell lines, Saos-2 cell line had the highest expression of IHH, PTCH1, and GLI1. The expressions of IHH, PTCH1, and GLI1 in several samples were constitutively higher when compared to the rest of the tumor samples. However, lack of expression of GLI1, IHH and PTCH1 was observed in some samples.

We observed a high positive association of GLI1, IHH, and PTCH1 with each other. The pairwise correlation coefficient between GLI1 and IHH was -0.442 (p=0.003), between GLI1 and PTCH1 was -0.751 (p<0.0001), and between IHH and PTCH1 was 0.560 (p=0.0001). The expression of PTCH1 was higher in tumor specimens that presented with metastasis at diagnosis. PTCH1 was the only gene that demonstrated statistically significant differential expression (p=0.003) between the two outcomes. Moreover, PTCH1 also demonstrated a significant hazard ratio of 0.725 (p=0.004) in disease-free survival analysis. The expression level of PTCH1, GLI1 and IHH was not associated with p53 mutation status, tumor size, necrosis, age, and gender.

DISCUSSION:
We have demonstrated that the expressions of IHH, GLI1 and PTCH1 in the Hedgehog pathway are positively correlated with each other. The expression of PTCH1 was higher in tumor specimens from patients that presented with metastasis at diagnosis.

Overexpression of genes may result in pathway deregulation, which may lead to the progression of osteosarcoma. One possible mechanism for overexpression of IHH and GLI1 genes might be gene amplification. GLI1 is upregulated in response to IHH signaling. Since transcription factor GLI1 mediates the transcription of downstream genes, overexpression of GLI1 may also induce overexpression of downstream genes including PTCH1. Real-time PCR is being utilized to determine if GLI1 and IHH DNA are amplified in osteosarcoma tumor samples and cell lines. Our data has shown that expression of the tumor suppressor gene, PTCH1, was less in tumors from patients who were metastatic at diagnosis (p<0.003). Lower levels could be due to mutations, and we are currently using Single Strand Conformation Polymorphism (SSCP) analysis and sequencing to screen for PTCH1 receptor mutations from frozen tissue specimens.

By drawing from the correlated expression of GLI1, PTCH1, and IHH in osteosarcoma, we can predict that blocking Indian Hedgehog signaling in osteosarcoma may lead to decreased cell growth. Osteosarcoma cell lines treated with cyclopamine are being used to further elucidate the phenotypic effect of blocking this pathway in vitro. This work may lead to novel molecular therapeutic strategies for osteosarcoma.

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