INTRODUCTION:
Each year, there are more than 800 new patients diagnosed with osteosarcoma under the age of 20 in the United State. Major progress has been made in the treatment of osteosarcoma patients due to the use of chemotherapy, leading to an improved overall survival rate of 65%. However, 30-40% of the patients with osteosarcoma still experience recurrence or metastasis despite the improved multimodality therapy. Recently, one of the most researched areas in sarcoma treatment is tyrosine kinases. Here, we describe research on a serine/threonine kinase, cyclin G-associated kinase (GAK), which has not been reported in osteosarcoma previously. In this study, we analyzed the expression of GAK in its function in osteosarcoma cell lines. Additionally, we hypothesized that GAK might have a role as a prognostic indicator for osteosarcoma. These data may contribute to the growing information of kinases for clinical utility in the treatment of osteosarcoma.

MATERIALS AND METHODS:
Cell cultures and tissues
Human osteosarcoma cell lines lines KHOS, U-2OS and the multidrug resistant (MDR) KHO52 were cultured in RPMI1640 with 10% FBS. Human osteoblast cells, HOB-c, were cultured in osteoblast growth medium with 10% FBS. Tissue samples were obtained from Massachusetts General Hospital sarcoma tissue bank, and were used in accordance with the policies of the institutional review board. Lentiviral human kinase shRNA library screen using MISSION™ LentExpress™ human kinases shRNA library
673 human kinases were analyzed for their effects on osteosarcoma cell growth using MISSION™ LentExpress™ Human Kinases shRNA Library (Sigma).

Synthetic GAK siRNA and transfection
GAK siRNA and non-specific siRNA were purchased from Ambion. The siRNA sequence targeting GAK (Genbank accession no. NM_005255.2) corresponded to coding regions (sense 5'-GUCUCGGCGUAUUAGGGAG-3', antisense 5'-UGCAUAAUUGAGCGAGCAG-3') of the GAK gene. Transfections were performed with Lipofectamine 1/4™ RNAIMAX (Invitrogen).

Immunofluorescence microscopy
Osteosarcoma cells were grown with either 100 nM GAK siRNA or non-specific siRNA on Lab-Tek™ chamber slides and fixed in 3.7% paraformaldehyde. Immunostainings were done using antibodies against GAK (1:50 dilution). The cells were incubated with Alexa Fluor secondary antibodies (Invitrogen). The nuclei were counterstained with 1 µg/mL Hoechst 33342.

Effect of GAK depletion on osteosarcoma cell proliferation
Proliferation of the cells was assessed using the CellTiter 96™ Aqueous One Solution Cell Assay (Promega).

Tissue microarray slide and immunohistochemistry
Osteosarcoma tissue microarrays were obtained from IMGENEX. The staining was preformed with HRP DAB Cell and Cell Tissue Staining Kit (R&D Systems). The primary antibody was applied at 4 °C overnight (1:50 dilution). Slide was counterstained with hematoxylin QS (Vector Laboratories) and mounted with VectorMount AQ.

Western blot analysis:
Cells were treated with increasing concentrations of GAK siRNA, non-specific siRNA or medium alone for 48h. Cell lysate was generated through RIPA Lysis Buffer (Upstate Biotechnology) and transferred to a PVDF membrane according to the standard technique. Immunoblotting was performed using antibodies against the indicated proteins.

RESULTS:
Identification of GAK as a new regulator of osteosarcoma cell survival
There were nine kinases, when knocked down, which displayed inhibitory growth effects on KHOS. Validation was done using shRNA clones targeting the kinase hits in a secondary osteosarcoma cell line, U-2OS. Although most of the kinases tested showed only limited effects, 3 out of 5 shRNA target sites of GAK inhibited osteosarcoma cell growth. GAK is overexpressed in osteosarcoma cell lines and tissues
Both drug sensitive and MDR osteosarcoma cell lines overexpressed GAK when compared to a normal human osteoblast. GAK was also expressed in all 6 osteosarcoma tissue samples from the patients. Confirmation of GAK knockdown using synthetic siRNA
The GAK siRNA was efficiently incorporated into osteosarcoma cell lines (Fig. 1). The results were also confirmed by western blot.

GAK depletion inhibits proliferation of osteosarcoma cell lines
Non-specific siRNA did not affect the growth, whereas 10 nM of GAK siRNA was enough to significantly decrease the cell proliferation in both drug sensitive and MDR osteosarcoma cell lines.

GAK and its correlation to osteosarcoma patient's survival
Kaplan-Meier survival analysis of osteosarcoma patients between the low-staining and the high-staining group from the tissue microarray showed that the prognosis for patients in the high-staining group was significantly worse than that of GAK low-staining group. GAK expression level did not show any significant differences between age, gender, or histological subtype.

Effect of GAK knockdown on signal transduction
GAK siRNA did not suppress the expression of either IGF-1 or EGF receptors and their phosphorylated forms, but interestingly, the expression of both receptors gradually increased along with the increase of GAK siRNA concentration. There was no change in Pgp expression even with the addition of 100 nM GAK siRNA. In contrast to the up-regulation of the receptors by GAK depletion, expressions of pAKT, pmTOR, and pSTAT3 were suppressed in a dose dependent manner.

DISCUSSION:
The importance of kinases has been established in many cancers, but their diversity of functions in osteosarcoma has yet to be elucidated. In this study, we first performed kinase shRNA screening which revealed that loss of function of GAK resulted in marked cell deaths of osteosarcoma cells. This result was further analyzed using GAK siRNA, and we showed that (1) GAK inhibition leads to less cell proliferation even in MDR cells, that (2) GAK expression correlates with worse outcome in osteosarcoma patients and can be a promising predictor of osteosarcoma prognosis and that (3) IGF-IR and EGFR, two receptors which have been repeatedly described in osteosarcoma, were up-regulated after GAK knockdown, but the downstream effectors such as AKT, mTOR, and STAT3 were significantly decreased in activities as shown by a decrease in phosphorylation for each protein. The results imply that GAK knockdown leads to defect of receptor trafficking which in part may be due to blockage of degradation of receptors during endocytosis leading to altered downstream signaling.

In summary, GAK is overexpressed in osteosarcoma cells and required for osteosarcoma cell proliferation. GAK exerts its effects by perturbing the tyrosine receptor trafficking and causes alterations of signal transductions. These findings may lead to development of new therapeutic options for osteosarcoma.