Cyclin G-associated kinase is necessary for osteosarcoma cell proliferation and receptor trafficking

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Running Title: Cyclin G-associated kinase and osteosarcoma

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Abbreviations List: GAK: cyclin G-associated kinase; IGF: insulin-like growth factor; EGF: epidermal growth factor; GIST: gastrointestinal stromal tumors; Hsc 70: heat shock cognate 70; IGF-IR: insulin-like growth factor 1 receptor; EGFR: epidermal growth factor receptor; MDR: multidrug resistant; FBS: fetal bovine serum; P-gp: p-glycoprotein; CCV: clathrin-coated vesicles; CCP: clathrin-coated pit.

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Abstract

Osteosarcoma is the most frequent primary malignant bone tumor among the children. The advent of neo-adjuvant chemotherapy significantly improved the prognosis of osteosarcoma patients in the 1980’s, but it has since plateaued in the past decades. 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, a lentiviral based human shRNA library was utilized to screen for kinases in KHOS and U-2OS osteosarcoma cells. The expression of GAK was examined in osteosarcoma and the effect on cell proliferation was analyzed by GAK siRNA knockdown. The level of GAK expression and its correlation to prognosis was analyzed in osteosarcoma tissue microarray. The effect of GAK depletion on insulin-like growth factor (IGF) and epidermal growth factor (EGF) receptor-mediated signal transduction was analyzed by western blot. We observed that GAK was overexpressed in both osteosarcoma cell lines and tissue samples when compared to human osteoblasts. GAK knockdown by siRNA decreased cell proliferation in both drug sensitive and multidrug resistant osteosarcoma cell lines. Immunohistochemistry of osteosarcoma tissue microarray revealed that overexpression of GAK was associated with poor prognosis. Finally, knockdown of GAK resulted in alterations of receptor trafficking and several downstream proteins. In conclusion, our results suggest that osteosarcoma cell proliferation and survival is dependent on GAK. These findings may lead to development of new therapeutic options for osteosarcoma.
Introduction

Osteosarcoma has the highest prevalence (approximately 60%) among patients with primary malignant bone tumors. Each year, there are more than 800 new patients diagnosed with osteosarcoma under the age of 20 in the United States (1). 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. Many will develop resistance to multiple types of chemotherapy after prolonged periods of treatments.

The importance of kinases has been established in many cancers, but their diversity of functions in osteosarcoma has yet to be elucidated. Nevertheless, targeting kinases has tremendous potential as shown in the treatment of gastrointestinal stromal tumors (GIST) treated with imatinib mesylate (Gleevec, Novartis Pharmaceuticals Corp.) (2).

Cyclin G associated kinase (GAK), also known as auxillin 2, is a 160-kDa serine/threonin protein kinase which was cloned from rats and humans in 1997 (3, 4). GAK is composed from N-terminal kinase domain that phosphorylates the µ-subunits of adaptor protein 1 and 2, clathrin-binding domain, and a C-terminal J-domain that interacts with heat shock cognate 70 (Hsc 70). Although it shares 43% homology to auxillin, which is only expressed in the neuronal cells (5), GAK is expressed ubiquitously in various organs. GAK is known to assemble clathrin into baskets during endocytosis and support Hsc 70 in uncoating clathrin-coated vesicles (6, 7). In yeast and C. elegans,
auxillin depletion has been reported to cause accumulation of clathrin coated vesicles, impaired cargo delivery to the vacuoles, and slow cell growth (8-10). GAK was identified in our lentiviral shRNA screen as being critical for osteosarcoma cell proliferation. Because GAK has important functions as a regulator of receptor tyrosine kinase trafficking, which is situated at the upstream of many kinases that has been reported to play key regulatory roles in the development of cancer, it was our aim to further examine its function in osteosarcoma cell lines.

In osteosarcoma, receptor tyrosine kinases such as insulin-like growth factor 1 receptor (IGF-IR) (11-15) and epidermal growth factor receptor (EGFR) (16-19) has been implicated in malignant growth and invasion. When ligands bind to each respective receptor, they are activated by autophosphorylation, internalized, at least in part through clathrin-mediated endocytosis, and sorted to early and late endosomes. Activation of signal transduction pathways and consequent subcellular reactions are mediated through the subcellular compartmentalization of each receptor tyrosine kinases. It is important to note that osteosarcoma is characterized by heterogeneity that has numerous genetic changes. Although there are several clinical trials currently undergoing that utilizes various drugs to block the IGF1R and EGFR signaling cascade (www.clinicaltrials.gov), it may be logical to do a sequential or combinatorial application of several kinase inhibitors for the treatment of osteosarcoma. Therefore, it may be useful to identify new kinases associated with osteosarcoma to develop appropriate treatments for diverse osteosarcoma patients.

In this study, we analyzed the expression of GAK and 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 culture and tissues

The human osteosarcoma cell line U-2OS was obtained from American Type
Culture Collection (Rockville, MD). Dr. Efstathios S. Gonos (National Hellenic
Research Foundation, Athens, Greece) kindly provided the human osteosarcoma cell line
KHOS and the multidrug resistant (MDR) cell lines KHOSR2 and U-2OSR2. All cell lines
were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS),
100 U/ml penicillin, and 100 ug/ml streptomycin (all obtained from Invitrogen, Carlsbad,
CA). Cells were incubated at 37°C in 5% CO2-95% air atmosphere and passaged when
near confluent monolayers were achieved using trypsin-EDTA solution. Resistant cell
lines were continuously cultured in 0.1 uM doxorubicin. Doxorubicin was obtained as
unused residual clinical material at the Massachusetts General Hospital. Human
osteoblast cells HOB-c were obtained from PromoCell GmbH (Heidelberg, Germany).
Osteoblast cells were cultured in osteoblast growth medium (PomoCell) with 10% FBS.
Cells were free of mycoplasma contamination as tested by MycoAlert(R)
MycoplasmaDetection Kit from Cambrex (Rockland, ME). Authentication of the cell
line was not performed. Osteosarcoma tissue samples were obtained from Massachusetts
General Hospital sarcoma tissue bank. Surgically treated patients diagnosed with
osteosarcoma were identified and utilized for the study and were used in accordance with
the policies of the institutional review board of the hospital. All diagnoses were confirmed by light microscopy and immunohistochemistry.

Lentiviral human kinase shRNA library screen using MISSION® LentiExpress™ human kinases shRNA library

673 human kinases were analyzed for their effects on osteosarcoma cell growth using MISSION® LentiExpress™ Human Kinases shRNA Library (Sigma, Saint Louis, MO) as described previously (20).

Synthetic GAK siRNA and transfection

Two different GAK siRNAs and one non-specific siRNA which has no significant homology to any known gene sequences from mouse, rat, or human were purchased from Ambion (Austin, Tx). The siRNA sequences targeting GAK (Genbank accession no. NM_005255.2) corresponded to coding regions (1: sense 5’-

GUCCGUCGCUAUUAGGCAtt-3’, antisense 5’-UGCAUAAUAGCGACGGACtg-3’, 2: sense 5’-CACCAGAAAUCAUAGACUtt-3’, antisense 5’-

AAGUCUAUGAUUUCUGGUGtt-3’) of the GAK gene. The siRNA oligonucleotides were dissolved in nuclease-free water at a concentration of 100 µM and kept at -20 °C until the following transfection experiments. Transfections were performed with Lipofectamine™ RNAiMAX according to the manufacturer’s instruction (Invitrogen). 100 nM mixtures of siRNA, transfection agent, and medium were subsequently diluted with the same medium and applied at the designated concentration to each well. Medium was replaced with RPMI 1640 supplemented with 10 % FBS 24 h after each transfection.
Immunofluorescence microscopy

Osteosarcoma cells were grown with addition of either 100 nM GAK siRNA 1 or 100 nM non-specific siRNA on eight-well Lab-Tek™ chamber slides (Thermo Fisher Scientific, Rochester, NY) for 24 h and fixed in 3.7% buffered paraformaldehyde. Immunostainings were done using antibodies against GAK (1:50 dilution; sc-7864, Santa Cruz Biotechnology, Santa Cruz, CA) and actin (1:200 dilution; Sigma-Aldrich, St. Louis, MO) overnight. After washing, the cells were incubated with Alexa Fluor secondary antibodies (Invitrogen). To counterstain the nuclei, the cells were incubated with PBS containing 1 µg/mL Hoechst 33342 (Invitrogen) for 15 min. Stained cells were then visualized on a Nikon Eclipse Ti-U fluorescence microscope (Nikon Instruments Inc., Melville, NY) equipped with a SPOT RT digital camera (Diagnostic Instruments Inc., Sterling Heights, MI).

Effect of GAK depletion on osteosarcoma cell proliferation

Proliferation of the cells was assessed using the CellTiter 96®Aqueous One Solution Cell Assay (Promega, Madison, WI) and by direct enumeration. Briefly, 1 × 10³ cells were plated per well in a 96 well plate for the CellTiter 96®Aqueous One Solution Cell Assay and 2×10³ cells were seeded in a 24 well plate for enumeration. After 24 h of incubation with increasing concentrations of GAK siRNA, 100 nM non-specific siRNA or medium alone, medium was replaced and the cells were allowed to grow for 6 to 7 days. GAK siRNA 1 was utilized for CellTiter 96®Aqueous One Solution Cell Assay and GAK siRNA 2 was applied for direct enumeration. The absorbance for CellTiter
96® Aqueous One Solution Cell Assay was read on a SPECTRA max Microplate Spectrophotometer (Molecular Devices, Silicon Valley, CA) at a wavelength of 490 nm. The cells were counted manually for enumeration under light microscopy using Trypan blue exclusion. Each experiment was performed in triplicate.

Tissue microarray slide and immunohistochemistry

Osteosarcoma tissue microarrays were obtained from the IMGENEX (San Diego, CA). The microarray was composed of 58 osteosarcoma tissues and included duplicate core biopsies (2 mm in diameter) from fixed, paraffin embedded tumors. 5 samples were lost during the staining process and 2 patients died of other causes, therefore were excluded from the analysis. Slide was baked at 62°C for 1 h, deparaffinized in xylene for 15 min, transferred through 100% ethanol for 5 minutes, and then rehydrated with graded ethanol. Endogenous peroxidase activity was quenched by 5 min incubation in 3% hydrogen peroxide (H₂O₂) in methanol. Rest of the staining was preformed with HRP-DAB Cell and Tissue Staining Kit (R&D Systems, Minneapolis, MN) according to the manufacturer’s instruction. Primary antibody was applied at 4 °C overnight (1:50 dilution, Santa Cruz Biotechnology) in 1% bovine serum albumin. Slide was counterstained with hematoxylin QS (Vector Laboratories, Burlingame, CA) and mounted with VectaMount AQ (Vector Laboratories) for long-term preservation.

Evaluation of immunohistochemical staining

The percentage of cells showing positive cytoplasmic staining for GAK was calculated by reviewing the entire spot. Based of the percentage of cells with positive
cytoplasmic staining, the staining patterns were classified into a four-grade scale: 0,
absence of cell staining; 1+, weak staining; 2+, moderate staining; 3+, intense staining
(20). For statistical analysis, osteosarcoma patients were sub grouped as either GAK low-
staining group (scale 0, 1) or high-staining group (scale 2, 3). Categorizing of
immunostaining intensity was completed by two independent observers. Discrepant
scores between the two observers were rescored to arrive at a single final score. Light
microscopic images were obtained using a Nikon Eclipse Ti-U fluorescence microscope
(Nikon Corp.) with SPOT RT digital camera (Diagnostic Instruments Inc.).

Western Blot analysis

Cells were seeded onto 6 well plates (4 × 10^5 cells/well). After 24 h of incubation
with increasing concentrations of GAK siRNA, non-specific siRNA or medium alone,
medium was replaced and the cells were allowed to grow for another 48 h. Protein
lysates from cells were generated through lysis with RIPA Lysis Buffer (Upstate
Biotechnology, Charlottesville, VA). The concentration of the protein was determined by
Protein Assay reagents (Bio-Rad, Hercules, CA) and a spectrophotometer (Beckman DU-
640, Beckman Instruments, Inc., Columbia, MD). Western blot analysis was performed
as previously reported (21). The mouse monoclonal antibody to GAK was purchased
from MBL International Corp. (Woburn, MA). Mouse monoclonal antibodies to EGFR
and pSTAT3 were purchased from Cell Signaling Technologies (Cambridge, MA).
Rabbit polyclonal antibodies to IGF-IR, pAKT, pmTOR, and pEGFR were purchased
from Cell Signaling Technologies as well. Pgp1 monoclonal antibody C219 was
purchased from Signet (Dedham, MA). The rabbit polyclonal antibody to human pIGF-
IR (1158/1162/1163) was purchased from Invitrogen. Goat antimouse-HRP and goat antirabbit-HRP were purchased from Bio-Rad (Hercules, CA). SuperSignal® West Pico Chemiluminescent Substrate was purchased from PIERCE (Rockford, IL). Bands were semi-quantified by reverse image scanning densitometry with PhotoShop 7.0 (Adobe, San Jose, CA).

**Statistical analysis**

Student’s t-test was used to compare the differences between groups (GraphPad PRISM®4 software, GraphPad Software, San Diego, CA). The Kaplan-Meier method was used to determine the relationship between GAK staining levels and patient survival, and data were analyzed with the log rank test (GraphPad PRISM®4 software). Results are given as mean ±SD and results with p<0.05 were considered statistically significant.

**Results**

**Identification of GAK as a new regulator of osteosarcoma cell survival**

We performed a screen that targeted 673 kinase genes by lentiviral shRNA and determined its effects on osteosarcoma cell survival. There were nine kinases, when knocked down, which displayed inhibitory growth effects on KHOS. We further validated these shRNA clones targeting the kinase hits in a secondary osteosarcoma cell line, U-2OS. Validated kinases included GAK, PLK1 (20), Mirk (22), and ROCK1. Although most of the kinases tested showed only limited effects, 3 out of 5 shRNA target sites of GAK inhibited osteosarcoma cell growth (Fig. 1). Molecular signaling has become increasingly important for the treatment of various cancers, and because GAK...
has been implicated as an important kinase in the trafficking of receptors which is at the very upstream of signal transduction, it was chosen to further investigate its functional role in osteosarcoma cell lines.

GAK is overexpressed in osteosarcoma cell lines and tissues

GAK protein expression was analyzed in several osteosarcoma cell lines and in osteosarcoma tissues by western blot. The results show that both drug sensitive and MDR osteosarcoma cell lines overexpressed GAK when compared to a normal human osteoblast cell line (Fig. 2A). To preclude the possibility that GAK expression is not only an artifact from prolonged culturing of the cell lines, 6 osteosarcoma tissue samples from patients were also analyzed. Samples 1 and 2 were taken prior to chemotherapy, and samples 3 to 6 were taken after chemotherapies were performed. GAK was expressed in all the samples tested (Fig 2B).

Confirmation of GAK knockdown using synthetic siRNA

To assess the transfection efficacy of GAK siRNA, two cell lines, KHOS and KHOSR2, were examined. Both GAK siRNA were efficiently incorporated into drug sensitive KHOS (Fig. 3A) and drug resistant KHOSR2 (Fig. 3B), and expression of GAK was suppressed at a minimum concentration of 10 nM (Fig. 3A). Immunofluorescence results further confirmed the knockdown of GAK in both drug sensitive (Fig. 3C) and MDR (Fig. 3D) osteosarcoma cell lines.

GAK depletion inhibits proliferation of osteosarcoma cell lines
To analyze the phenotype of GAK knockdown on osteosarcoma cell lines, CellTiter 96® Aqueous One Solution Cell Assay and direct enumeration using Trypan blue exclusion was performed. The absorbance for CellTiter 96® Aqueous One Solution Cell Assay showed that non-specific siRNA did not affect the growth of both drug sensitive and MDR osteosarcoma cell lines, whereas 10 nM of GAK siRNA 1 was enough to significantly decrease the cell proliferation in both drug sensitive KHOS (Fig. 4A) and MDR KHOSR2 (Fig. 4B) osteosarcoma cell lines (p<0.05). Similarly, direct enumeration of the osteosarcoma cell lines under light microscopy after inhibition of GAK with GAK siRNA 2 showed decreased cell proliferation with minimum cell deaths for both KHOS (Fig. 4C) and KHOSR2 (Fig. 4D).

GAK and its correlation to osteosarcoma patient’s survival

We further analyzed GAK expression by immunohistochemistry using tissue microarray (Fig. 5A-C). Patient prognosis was compared with expression levels of GAK. Intensity of GAK staining was compared between samples from survivors (survival at 60 months follow-up) and non-survivors (Died in <60 months follow-up). In 26 non-survivors, 18 out 26 (69.2%) expressed GAK at high levels (>2+), whereas in 25 survivors, 4 out 25 (16.0%) expressed GAK in high levels (Table 1). The average expression level of GAK staining for survivors and non-survivors were 1.4 and 2.1, respectively (Fig. 5D). The level of GAK staining for samples from non-survivors was significantly higher than that for survivors (p=0.0024). Kaplan-Meier survival analysis of osteosarcoma patients between the low-staining and the high-staining group showed that the prognosis for patients in the high-staining group was significantly worse than that
of GAK low-staining group (Fig. 5E). GAK expression level did not show any significant differences between age, gender, or histological subtype (data not shown).

Effect of GAK knockdown on receptors and downstream effectors of osteosarcoma

GAK is a potential regulator of clathrin-mediated membrane trafficking of various receptors. To examine the effect of GAK depletion on IGF-IR and EGFR signal transduction, western blot analysis was performed. GAK siRNA did not suppress the expression of either IGF-I or EGF receptors and their phosphorylated forms, but interestingly, the expression of both receptors gradually increased along with the increase of GAK siRNA concentration (Fig. 6A, Supplemental Fig. 1). The same effect was observed in both drug sensitive and drug resistant osteosarcoma cell lines. P-glycoprotein (P-gp) has been implicated as one of the major cause in the drug resistance of various cancers (23) and in osteosarcoma (24). Effect of GAK knockdown on P-gp expression was also analyzed to see whether GAK has any effect on the P-gp induction in drug resistant osteosarcoma cells. There was no change in P-gp expression even with the addition of 100 nM GAK siRNA. To assess the effect of GAK knockdown on the downstream signaling cascade of IGF-IR and EGFR, expressions of pAKT, pmTOR, and pSTAT3 were analyzed. In contrast to the up-regulation of pIGF-IR and EGFR by GAK depletion, expressions of pAKT, pmTOR, and pSTAT3 were suppressed in a dose dependent manner (Fig. 6B).

Discussion
In this study, we first performed kinase shRNA screening which revealed that loss of function of GAK, among others, resulted in marked growth inhibition of osteosarcoma cells. High throughput screening of the kinome is a powerful tool where one can identify multiple kinases related to survival of cancer cells. We have previously reported on other kinases - PLK1 (20) and Mirk (22) - in osteosarcoma which was discovered using the same lentiviral based shRNA library. Currently, PLK1 inhibitors are already in clinical trial for several cancers (25-28). Theoretically, the best gene for targeted therapy would be ones that are highly expressed in tumor cells while minimally expressed in the normal tissues. In contrast to the high expression of GAK in osteosarcomas, normal human osteoblasts only expressed low quantity of the protein. The result of kinase shRNA screening was further confirmed by siRNA knockdown of GAK on several osteosarcoma cell lines. Although 100 nM non-specific siRNA did not have any cytotoxic activity on osteosarcoma cell lines, a concentration of as low as 10 nM of GAK siRNA was enough to inhibit the proliferation of osteosarcoma cells. Importantly, it had similar effects on both drug sensitive and drug resistant osteosarcoma cell lines which implicate that it exerts its effects independently of ATP-binding cassette transporters such as P-gp. This was further confirmed by western blot analysis of P-gp which did not show any effect on P-gp trafficking. Currently, extensive research is underway to develop novel agents to overcome the drug resistance of osteosarcoma, but the results have been unsuccessful to date, hampered by low efficiency and/or high toxicity (29-32). GAK has the potential to be a target for the treatment of drug naïve osteosarcomas as well as MDR osteosarcomas. GAK was originally identified as a protein that can be immunoprecipitated with cyclin G (3). Subsequent assays demonstrated that GAK does not regulate cell cycle, but
it is present in clathrin-coated vesicles (CCV) and that kinase domain of GAK was
directed toward the µ2 subunit of adaptor protein 2 in CCVs (33). Clathrin-mediated
endocytosis is one of the mechanisms where various receptors are internalized as cargos.
CCVs arise as a result of clathrin-coated pit (CCP) invagination and constriction. GAK
and the molecular chaperone Hsc 70 have been reported to be involved in constriction of
the vesicle neck and scission of the CCVs from the membrane (34). GAK is also
reported to have a role in assembling clathrin triskelions into clathrin baskets and is
known to interact with Hsc 70 in the uncoating of clathrin from CCVs (6, 7, 35, 36). The
J domain of GAK binds to Hsc 70 and stimulates its ATPase activity which destabilizes
the clathrin-clathrin interactions in CCVs (37). Although GAK’s functional mechanism
has been extensively studied, its implication on cancer has not been determined. It has
been suggested that the effect of GAK down-regulation vary between different receptors
and between different cell lines. Down-regulation of GAK has been reported to
transform CV1P (African green monkey kidney cell line) cells into highly proliferating
cells (38). Similarly, GAK knockdown in Hela cells (Human cervix epithelioid
carcinoma cell line) resulted in promotion of cell proliferation (38). In contrast, GAK
knockdown lead to cell death in myeloma tumor cells as a part of results of high
throughput kinome siRNA screening (39). GAK expression has been implicated as a
prognostic biomarker in prostate cancers also, where GAK expression increased with
prostate cancer progression to androgen independence (40). Our results with
osteosarcoma are compatible with myeloma and prostate cancer where GAK inhibition
led to decreased cell proliferation.
There is scant data regarding pro-oncogenic function of GAK in tumors, but immunohistochemical staining of osteosarcoma tissues suggest that there is an association between GAK expression levels and sarcoma progression. Although we could not find any correlation between age, gender, primary site, or histological subtype based on our limited number of samples, further studies using greater number of patient samples are warranted. While the exact function of GAK overexpression in advanced disease is still not clear, it has been suggested that GAK overexpression correlates with increased responsiveness of receptors in prostate cancers as they progress in malignancy (40). Our results also suggest that GAK expression correlates with worse outcome in osteosarcoma patients and can be a promising predictor of osteosarcoma prognosis.

GAK depletion has been linked to alterations of various receptor tyrosine kinase trafficking and signaling. GAK knockdown caused EGFR up-regulation in HeLa cells, but did not cause any change in HER2 nor IGFR (38). The same group did not observe any change in PDGFR expression upon inhibition of GAK in NIH3T3 cells. Depletion of GAK on various downstream signaling has been reported as well with conflicting results. Zhang et al (37) reported that transient depletion of GAK by siRNA in Hela cells caused defects in transferrin uptake and procathepsin D to cathpesin D maturation suggesting a defect in trans-Golgi to endosome trafficking. GAK knockdown by siRNA in HeLa cells resulted in increased activation of ERK1/2, ERK5, and AKT (38) whereas introduction of dominant-negative dynamin, another protein involved in receptor trafficking, inhibited MAPK activation (41). GAK depletion has been linked to decrease in expression of Her4, a Notch target gene, in zebrafish models (42). Two receptors which have been repeatedly described in osteosarcoma, IGF-IR and EGFR, were analyzed to assess the effect of GAK.
depletion on their expression. In our study, both receptors were up-regulated after GAK knockdown by siRNA. While there are multitudes of different kinases involved in the signal transduction of IGF-IR and EGFR, downstream effectors such as AKT, mTOR, and STAT3 were significantly decreased in activities as shown by a decrease in phosphorylation for each protein after GAK suppression. The difference from the previous studies may be due to the difference in signal transduction of our receptor tyrosine kinases in osteosarcoma compared to other cell types. 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. Our results suggest that GAK has profound effect on receptor trafficking and signaling in osteosarcoma and may be a potential target in therapy.

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.
References


Table 1  Distribution of GAK staining level and its correlation to survival of osteosarcoma patients.

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Figure Legends

Figure 1 - Results of lentiviral shRNA knockdown in KHOS osteosarcoma cells.
Each data represents a well from 96-well plate of MISSION® LentiExpress™ Human kinases shRNA library. The effects of GAK knockdown in osteosarcoma cell lines were assessed using 5 different sequences of GAK gene. Three out of 5 sequences for GAK shRNA (lanes 2, 3, and 5) effectively inhibited the growth of osteosarcoma cells compared to the negative controls (lanes 6 and 7). Medium-only environment which contains untransduced cells and causes complete cell death in 7 days, was used as a positive control (lane 8).

Figure 2 - GAK is expressed in osteosarcoma. Osteosarcoma cell lines KHOS, KHOSR2, U-2OS, and U-2OSR2 overexpressed GAK when compared to a normal osteoblast cell line HOB-c (Fig. 2A). All the osteosarcoma tissues examined expressed GAK (Fig. 2B). Lanes 1-6 represent six different patients’ tissue samples. Tissue samples for lanes 1 and 2 were taken prior to treatment, and samples 3 to 6 were taken after chemotherapies were performed.

Figure 3 - Effective knockdown of GAK by siRNA. GAK protein expression was effectively inhibited with two sequences of GAK siRNA at a minimal concentration of 10 nM in both drug sensitive KHOS (Fig. 3A) and drug resistant KHOSR2 (Fig. 3B) cell lines (M: medium, c-siRNA: 100 nM non-specific siRNA). The results of the
knockdown of GAK in both KHOS (Fig. 3C) and KHOSR2 (Fig. 3D) were further
confirmed by immunofluorescence microscopy. GAK (green) was localized using anti-
GAK antibodies and then stained with Alexa Fluor secondary antibody.

**Figure 4 - Effect of GAK depletion on osteosarcoma cell lines.** KHOS and KHOSR2
were transfected with respective siRNA or medium alone, and cell proliferation after
transfection was determined by CellTiter 96®Aqueous One Solution Reagent. Addition
of varying concentrations of GAK siRNA 1 showed decreased proliferation (*p<0.05) as
compared to medium alone and 100 nM non-specific siRNA for both KHOS (Fig. 4A)
and KHOSR2 (Fig. 4B) (mean ± SD). Additionally, direct enumeration of the
osteosarcoma cells with Trypan blue exclusion after inhibition of GAK with GAK siRNA
2 showed decreased cell proliferation with minimum cell deaths for both KHOS (Fig. 4C)
and KHOSR2 (Fig. 4D) (mean ± SD). The experiment was repeated three times in
triplicate.

**Figure 5 - Correlation of GAK low-staining and high-staining on osteosarcoma
patient survival.** Osteosarcoma tissue microarray consisting of 51 samples was stained
using antibodies against GAK. The staining of each tissue samples were classified into
four-grade scale: 0, absence of cell staining; 1+, weak staining; 2+, moderate staining;
3+, intense staining. (A-C) Representative pictures of immunohistochemical staining of
tissue microarray. Left panels: HE stainings. Right panels: Weak-staining (1+, A),
moderate-staining (2+, B), and high-staining (3+, C) pictures of GAK expression in
osteosarcoma tissue samples. (D) Distribution of GAK staining scores among the
survivors (survival at 60 months follow-up) and non-survivors (Died in <60 months follow-up) of osteosarcoma. There was significant difference in staining scores between the survivors and the non-survivors (p=0.0024). (E) Kaplan-Meier survival curve of GAK low-staining and high-staining patient samples. For analysis, patients were grouped into either GAK low-staining group (scale 0, 1) or high-staining group (scale 2, 3). The prognosis of GAK low-staining group was significantly better than the high-staining group (p=0.001).

**Figure 6 - Western blot analysis was performed to assess the effect of GAK depletion on osteosarcoma receptor expressions and downstream signaling.** (A) The expression of both pIGF-IR and pEGFR increased along with the increase in the concentration of GAK siRNA 1. While the expressions of IGF-IR and EGFR were slightly increased or unchanged in KHOSR2, there was significant increase in both receptors in KHOS after GAK inhibition with 100 nM GAK siRNA. GAK depletion had no effect on the expression of P-gp. (B) Expressions of pAKT, pmTOR, and pSTAT3 which are downstream effectors of growth factor receptor signalings, were decreased as the concentration of GAK siRNA increased. The analysis was repeated three times.
1: GAK lentiviral shRNA particle(x-1244s1c1)
2: GAK lentiviral shRNA particle(x-3438s1c1)
3: GAK lentiviral shRNA particle(x-1870s1c1)
4: GAK lentiviral shRNA particle(x-2091s1c1)
5: GAK lentiviral shRNA particle(x-4100s1c1)
6: vector control(SHCOO1V)
7: Non-specific shRNA(SHCOO2V)
8: Medium control

Figure 1
Figure 2

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<td>GAK</td>
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<td>β-actin</td>
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Figure 3
Figure 4: Temperature Stability of miRNA with Different Titrations in KHOS and KHOS R2

A. Absorbance (490nm) over Days 2 to 6 for KHOS Medium and KHOS 100nM c-siRNA, KHOS 10nM GAK-siRNA, KHOS 30nM GAK-siRNA, and KHOS 100nM GAK-siRNA.

B. Absorbance (490nm) over Days 2 to 7 for KHOS R2 Medium, KHOS R2 100nM c-siRNA, KHOS R2 10nM GAK-siRNA, KHOS R2 30nM GAK-siRNA, and KHOS R2 100nM GAK-siRNA.

C. Cell number over Days 1 to 7 for KHOS Medium, c-siRNA, 10nM GAK, 30nM GAK, and 100nM GAK.

D. Cell number over Days 1 to 7 for KHOS R2 Medium, c-siRNA, 10nM GAK, 30nM GAK, and 100nM GAK.
Figure 6

A. KHOS and KHOS\textsubscript{R2} cells treated with GAKsiRNA1 and siRNA at different concentrations (M, c-siRNA, 10nM, 30nM, 100nM) were analyzed for expression of IGF-IR, pIGF-IR, EGFR, pEGFR, Pgp, β-actin.

B. KHOS and KHOS\textsubscript{R2} cells treated with GAKsiRNA1 and siRNA at different concentrations (M, c-siRNA, 10nM, 30nM, 100nM) were analyzed for expression of pAKT, pmTOR, pSTAT3, β-actin.
Cyclin G-associated kinase is necessary for osteosarcoma cell proliferation and receptor trafficking

Michiro Susa, Edwin Choy, Xianzhe Liu, et al.

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