Preclinical Development

Cyclin G–Associated Kinase Is Necessary for Osteosarcoma Cell Proliferation and Receptor Trafficking

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Abstract

Osteosarcoma is the most frequent primary malignant bone tumor among the children. The advent of neoadjuvant chemotherapy significantly improved the prognosis of patients with osteosarcoma in the 1980s, 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 and epidermal growth factor 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 with 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 are dependent on GAK. These findings may lead to the 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, younger than 20 years, with a diagnosis of osteosarcoma in the United States (1). Major progress has been made in the treatment of patients with osteosarcoma because of the use of chemotherapy, leading to an improved overall survival rate of 65%. However, 30% to 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 treated with imatinib mesylate (Gleevec; Novartis Pharmaceuticals Corp; ref. 2).

Cyclin G–associated kinase (GAK), also known as auxillin 2, is a 160-kDa serine/threonin protein kinase that was cloned from rats and humans in 1997 (3, 4). It 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 expressed only in the neuronal cells (5), GAK is expressed ubiquitously in various organs. 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 have 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; refs. 11–15) and epidermal growth factor receptor (EGFR; refs. 16–19) have 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 cellular reactions are mediated through the subcellular compartmentalization of each receptor tyrosine kinase. It is important to note that osteosarcoma is characterized by heterogeneity that has numerous genetic changes. Although there are several clinical trials (www.clinicaltrials.gov) currently undergoing that utilize various drugs to block the IGF-IR and EGFR signaling cascade, it may be logical to do a sequential or combinational 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 treatment options for diverse patients with osteosarcoma.

In this study, we analyzed the expression of GAK and its function in osteosarcoma cell lines. In addition, 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. Dr Efstathios S. Gonos (National Hellenic Research Foundation) kindly provided the human osteosarcoma cell line KHOS and the multidrug-resistant cell lines KHOS_{Rx} and U-2OS_{Rx}. All cell lines were cultured in RPMI 1640 medium supplemented with 10% FBS, 100 U/mL of penicillin, and 100 μg/mL of streptomycin (all obtained from Invitrogen). 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 μmol/L of doxorubicin. Doxorubicin was obtained as unused residual clinical material from the Massachusetts General Hospital. Human osteoblast cells HOB-c were obtained from PromoCell GmbH. Osteoblast cells were cultured in osteoblast growth medium (PromoCell GmbH) with 10% FBS. Cells were free of mycoplasma contamination as tested by MycoAlert Mycoplasma Detection Kit from Cambrex. Authentication of the cell line was not done. Osteosarcoma tissue samples were obtained from Massachusetts General Hospital sarcoma tissue bank. Surgically treated patients with a diagnosis of 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**

A total of 673 human kinases were analyzed for their effects on osteosarcoma cell growth, using MISSION LentiExpress Human Kinases shRNA Library (Sigma) as described previously (20).

**Synthetic GAK siRNA and transfection**

Two different GAK siRNAs and 1 nonspecific siRNA that has no significant homology to any known gene sequences from mouse, rat, or human were purchased from Ambion. The siRNA sequences targeting GAK (GenBank accession No. NM_005255.2) corresponded to coding regions (1: sense 5’-GUCCGUCGC-UAAUUAUGCAT-3’, antisense 5’-UGCAUAAUUAG-CGACGGAGct-3’; 2: sense 5’-CACCAGAAAUCAAGACUUtt-3’, antisense 5’-AGUCUAGAUUUUCCGUGtt-3’) of the GAK gene. The siRNA oligonucleotides were dissolved in nuclease-free water at a concentration of 100 μmol/L and kept at −20°C until the following transfection experiments. Transfections were performed with Lipofectamine RNAiMAX according to the manufacturer’s instruction (Invitrogen). Mixtures (100 nmol/L) of siRNA, transfection agent, and medium were subsequently diluted with the same medium and applied at the designated concentration to each well. The medium was replaced with RPMI 1640 supplemented with 10% FBS 24 hours after each transfection.

**Immunofluorescence microscopy**

Osteosarcoma cells were grown with the addition of either 100 nmol/L of GAK siRNA1 or 100 nmol/L of nonspecific siRNA on 8-well Lab-Tek chamber slides (Thermo Fisher Scientific, Rochester, NY) for 24 hours and fixed in 3.7% buffered paraformaldehyde. Immunostainings were done using antibodies against GAK (GenBank accession No. NM_005255.2) corresponding to coding regions (1: sense 5’-GUCCGUCGC-UAAUUAUGCAT-3’, antisense 5’-UGCAUAAUUAG-CGACGGAGct-3’; 2: sense 5’-CACCAGAAAUCAAGACUUtt-3’, antisense 5’-AGUCUAGAUUUUCCGUGtt-3’) of the GAK gene. The siRNA oligonucleotides were dissolved in nuclease-free water at a concentration of 100 μmol/L and kept at −20°C until the following transfection experiments. Transfections were performed with Lipofectamine RNAiMAX according to the manufacturer’s instruction (Invitrogen). Mixtures (100 nmol/L) of siRNA, transfection agent, and medium were subsequently diluted with the same medium and applied at the designated concentration to each well. The medium was replaced with RPMI 1640 supplemented with 10% FBS 24 hours after each transfection.

**Effect of GAK depletion on osteosarcoma cell proliferation**

Proliferation of the cells was assessed using the Cell-Titer 96 Aqueous One Solution Cell Assay (Promega) and by direct enumeration. Briefly, 1 × 10⁴ cells were plated per well in a 96-well plate for the CellTiter 96 Aqueous One Solution Cell Assay and 2 × 10⁴ cells were seeded in
a 24-well plate for enumeration. After 24 hours of incubation with increasing concentrations of GAK siRNA, 100 nmol/L of nonspecific siRNA or medium alone, medium was replaced and the cells were allowed to grow for 6 to 7 days. GAK siRNA1 was utilized for CellTiter 96 Aqueous One Solution Cell Assay and GAK siRNA2 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) at a wavelength of 490 nm. The cells were counted manually for enumeration by 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. The microarray was composed of 58 osteosarcoma tissues and included duplicate core biopsies (2 mm in diameter) from fixed, paraffin-embedded tumors. Five samples were lost due to other causes and therefore were excluded from the analysis. Slide was baked at 62°C for 1 hour, deparaffinized in xylene for 15 minutes, transferred through 100% ethanol for 5 minutes, and then rehydrated with graded ethanol. Endogenous peroxidase activity was quenched by 5-minute 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) according to the manufacturer’s instruction. Primary antibody was applied at 4°C overnight (1:50 dilution; Santa Cruz Bio-technology) in 1% bovine serum albumin. Slide was counterstained with hematoxylin QS (Vector Laboratories) for long-term preservation. Western blot analysis was performed as described in methods, using a Nikon Eclipse Ti-U fluorescence microscope (Nikon Corp.) with a SPOT RT digital camera (Diagnostic Instruments Inc.). Western blot analysis was performed as described. Bands were semiquantified by reverse image scanning densitometry with PhotoShop 7.0 (Adobe).

**Statistical analysis**

The Student t test was used to compare the differences between groups (GraphPad PRISM 4 software; GraphPad Software). 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 9 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 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 that 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 both in several osteosarcoma cell lines and in osteosarcoma tissues by Western blot. The results show that both drug-sensitive
and multidrug-resistant osteosarcoma cell lines overexpressed GAK when compared with a normal human osteoblast cell line (Fig. 2A). To preclude the possibility that GAK expression is not just 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, 2 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 nmol/L (Fig. 3A). Immunofluorescence results further confirmed the knockdown of GAK in both drug-sensitive (Fig. 3C) and multidrug-resistant (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 nonspecific siRNA did not affect the growth of both drug-sensitive and multidrug-resistant osteosarcoma cell lines, whereas 10 nmol/L of GAK siRNA1 was enough to significantly decrease the cell proliferation in both drug-sensitive KHOS (Fig. 4A) and multidrug-resistant KHOSR2 (Fig. 4B) osteosarcoma cell lines ($P < 0.05$).

Similarly, direct enumeration of the osteosarcoma cell lines by light microscopy after inhibition of GAK with GAK siRNA2 showed decreased cell proliferation with minimum cell deaths for both KHOS (Fig. 4C) and KHOSR2 (Fig. 4D).

GAK and its correlation to the survival of patients with osteosarcoma

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 nonsurvivors (died during <60 months follow-up). In 26 nonsurvivors, 18 of 26 (69.2%) patients expressed GAK at high levels (2+), whereas in 25 survivors, 4 of 25 (16.0%) patients expressed GAK in high levels (Table 1). The average expression level of GAK staining for survivors and nonsurvivors were 1.4 and 2.1, respectively.
The level of GAK staining for samples from nonsurvivors was significantly higher than that for survivors ($P = 0.0024$). Kaplan-Meier survival analysis of patients with osteosarcoma between the low-staining and the high-staining groups showed that the prognosis for patients in the high-staining group was significantly worse than that in the 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-IR or EGFR and their phosphorylated forms, but, interestingly, the expression of both receptors gradually increased along with the increase in GAK siRNA concentration (Fig. 6A; Supplementary Fig. S1). 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 causes of drug resistance in 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 nmol/L of GAK siRNA. To assess the effect of GAK knockdown on the downstream signaling cascade

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<tr>
<td></td>
<td>Survivors, %</td>
<td>Nonsurvivors, %</td>
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<tr>
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of IGF-IR and EGFR, expressions of pAKT, pmTOR, and pSTAT3 were analyzed. In contrast to the upregulation 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 that revealed that the 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 with which one can identify multiple kinases related to the survival of cancer cells. We have previously reported on other kinases in osteosarcoma—PLK1 (20) and Mirk (22)—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 expressed only 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 nmol/L of nonspecific siRNA did not have any cytotoxic activity on osteosarcoma cell lines, a concentration of as low as 10 nmol/L 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-naive osteosarcomas as well as multidrug-resistant osteosarcomas.

GAK was originally identified as a protein that can be immunoprecipitated with cyclin G (3). Subsequent assays showed that GAK does not regulate cell cycle, but it is present in CCVs and that kinase domain of GAK was directed toward the \( \mu_2 \)-subunit of adaptor protein 2 in CCVs (33). Clathrin-mediated endocytosis is one of the mechanisms in which various receptors are internalized as cargos. CCVs arise as a result of clathrin-coated pit invagination and constriction. GAK and the molecular chaperone Hsc 70 have been reported to be involved in the 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

Figure 6. Western blot analysis was performed to assess the effect of cyclin G–associated kinase (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. While the expressions of IGF-IR and EGFR were slightly increased or unchanged in KHOS\(_{R2}\), there was significant increase in both receptors in KHOS after GAK inhibition with 100 nmol/L of 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 signaling, decreased as the concentration of GAK siRNA increased. The analysis was repeated 3 times.
interactions in CCVs (37). Although the functional mechanism of GAK has been extensively studied, its implication on cancer has not been determined. It has been suggested that the effect of GAK downregulation varies between different receptors and between different cell lines. Downregulation 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 the promotion of cell proliferation (38). In contrast, GAK knockdown leads 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, in which GAK expression increases with prostate cancer progression to androgen independence (40). Our results of osteosarcoma are compatible with myeloma and prostate cancer for which GAK inhibition leads 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 a greater number of patient samples are warranted. Although 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 patients with osteosarcoma and can be a promising predictor of osteosarcoma prognosis.

GAK depletion has been linked to alterations in various receptor tyrosine kinase trafficking and signaling. According to Zhang et al., GAK knockdown caused EGFR upregulation in HeLa cells but did not cause any change in either HER2 or IGF receptor (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 cathepsin D maturation, suggesting a defect in the trans-Golgi network 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 (IGF-IR and EGFR) that have been repeatedly described in osteosarcoma were analyzed to assess the effect of GAK depletion on their expression. In our study, both receptors were upregulated after GAK knockdown by siRNA. Although 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 with other cell types. The results imply that GAK knockdown leads to defect of receptor trafficking, which, in part, may be due to the 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 in signal transductions. These findings may lead to the development of new therapeutic options for osteosarcoma.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References


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