The kinase Mirk is a potential therapeutic target in osteosarcoma

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Osteosarcoma is the most common primary malignant bone tumor affecting children and adolescents. The majority of patients are treated by surgery and chemotherapy but have limited alternative therapeutic options. Kinases play an important role in the growth and survival of tumor cells. We aim to identify specific kinases to be vital in the survival of osteosarcoma cells and thus may be a key target in creating novel anticancer therapies. A lentiviral short hairpin RNA kinase library, screened osteosarcoma cells, identified kinase minibrain-related kinase (Mirk) (Dyrk1B) as a potential target. Knockdown Mirk expression could inhibit cell growth and induce apoptosis. Chemically synthetic small interfering RNA knockdown and complementary DNA rescue assay further confirmed the results from the decrease of Mirk gene expression. The relationship between Mirk gene expression and the clinical characteristics of patients with osteosarcoma was investigated using tissue microarray and immunohistochemistry analysis. The data indicate that the overall survival rate of patients with Mirk high staining (high levels of Mirk protein expression) is significantly shorter than those with Mirk low staining and moderate staining. This highlights Mirk’s potential to serve as a promising target for molecular therapy in the treatment of osteosarcoma.

Introduction

Osteosarcoma is the most common primary malignant tumor of bone with high metastatic potential (1,2). Treatment of osteosarcoma requires therapy incorporating surgery and systemic chemotherapy. Chemotherapy protocols involve several chemotherapeutic agents, including doxorubicin, cisplatin, ifosfamide and methotrexate (3). However, if these agents are unable to solicit a favorable response, then any further therapeutic options are limited. One-third of patients with localized osteosarcoma experience recurrent or progressive disease (4) and the average survival period after a recurrence is <1 year (5). Therefore, to improve the survival rate of osteosarcoma patients and to better their overall well being, it is imperative to continuously develop novel therapeutic strategies.

Recently, research on osteosarcoma has been focused on identifying novel therapeutic targets and prognostic markers. Several molecular targets are currently under evaluation for osteosarcoma, including insulin-like growth factor 1 receptor, epidermal growth factor receptor, signal transducer and activator of transcription 3 and mammalian target of rapamycin (6–8). There is, however, insufficient data to allow any of these targets to be recommended as prognostic factors or therapeutic targets. One method for the identification of novel prognostic or therapeutic targets is utilizing a RNA interference screen.

Materials and methods

Cell lines and cell culture

Dr Efstathios Gonas (Institute of Biological Research and Biotechnology, Athens, Greece) provided the osteosarcoma KHOS cell line (originally from American Type Culture Collection) (24). Dr Katia Scotlandi (Institute Orthopedics Rizzoli, Italy) provided Ewing sarcoma cell line TC-71. The human osteosarcoma cell lines, U-2OS and Saos, uterine sarcoma cell line MES-SA and ovarian cancer cell line SKOV-3 were obtained from the American Type Culture Collection (Rockville, MD). Osteosarcoma cell line OSA344, chondrosarcoma cell line CS-1 and synovial sarcoma cell line SS-1 were established from primary sarcoma tissue in our laboratory. Human osteoblast cells HOb-c were obtained from PromoCell GmbH (Heidelberg, Germany), osteoblast cells NHOst were obtained from Lonza Walkersville (Walkersville, MD), and osteoblast cells hFOB were obtained from American Type Culture Collection. Osteoblast cells were cultured in osteoblast growth medium (PomoCell) with 10% fetal bovine serum. All other cell lines were cultured in RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen). Cells were incubated at 37°C in 5% CO2–95% air atmosphere and passaged when near confluent monolayers were achieved using trypsin–ethylenediaminetetraacetic acid solution.

Lentiviral human kinase shRNA library screen

The role of protein kinases in maintaining or supporting osteosarcoma cell growth was examined using MISSION® Lentivexpress™ Human Kinases shRNA library (Sigma, St Louis, MO). Transduction conditions in KHOS was optimized using MISSION® Non-target shRNA Control Transduction particles (SHC002V) (Sigma) in determining its efficiency as a negative control or therapeutic target. RNA interference suppresses gene expression in mammalian cells, and chemically synthesized small interference RNAs (siRNAs) have become essential tools for biological studies. Indeed, screens done in human cells using libraries of synthetic siRNAs targeting defined gene families have identified various kinases required for growth, survival and drug resistance in human cancer cells (9,10). This powerful new technique is also applicable to osteosarcoma cell lines in order to identify cellular signaling pathways that may be essential for osteosarcoma cell growth and survival and ultimately may be targets for novel therapy.

The human kinase contains at least 600 protein kinases that phosphorylate proteins at 250 000 or more sites (11–13). Kinases are dysregulated in many cancers, including osteosarcoma. Given that protein phosphorylation regulates cancer cell survival, strategies for targeting kinases are paramount for improved therapeutic intervention (13). It has been shown that the suppression of some kinases, such as tyrosine kinases Bcr-abl and Her2 (14,15), and the serine/threonine kinases Raf, Akt and mammalian target of rapamycin, inhibit tumor cell growth and proliferation, suggesting that development of inhibitors that target these kinases may lead to new anticancer strategies (16–18). The role of kinases in osteosarcoma is not currently well understood, and a thorough study of these proteins and their functions is probably to contribute to the discovery and development of new therapeutic approaches. Kinases, such as insulin-like growth factor 1 receptor, phosphoinositide 3-kinases/protein kinase B, platelet-derived growth factor receptor and mammalian target of rapamycin, have been found to be highly expressed in different sarcomas, particularly in the advanced stages (19–23), and identification of novel kinases whose inhibition induces osteosarcoma cell lethality would be of high value in clinical management.

In the present study, the roles of protein kinases in supporting osteosarcoma cell growth are examined using a human kinase short hairpin RNA (shRNA) library. Our screens elucidate that decreased expression of minibrain-related kinase (Mirk) (Dyrk1B) can inhibit growth and induce apoptosis in osteosarcoma cells. Additionally, we observe a high endogenous level of Mirk expression in osteosarcoma cell lines and osteosarcoma tissue. The relative level of Mirk expression in tumors is closely correlated with poor prognosis in patients.

Abbreviations: cDNA, complementary DNA; Mirk, minibrain-related kinase; siRNA, small interference RNA; shRNA, short hairpin RNA.

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control, optimal puromycin concentrations for selection and assay times prior to the kinase panel screen. Optimization of cell density and multiplicity of infection was determined in KHOS using MISSION® LentiExpress™ Optimization Plate (SHX01) with GFP lentiviral shRNA as a positive control for experiment. After optimizing for cell seeding density, concentration of puromycin and time points, the effects of kinase knockdown by lentiviral shRNA were carried out with the LentiExpress™ Human Kinases shRNA library. In brief, on day 1, KHOS cells were diluted to 40,000 cells/ml in complete medium. Polybrene (hexadimethrine bromide) was added to a final concentration of 11.3 µg/ml and then 70 µl of cell suspension was added to each well of a LentiExpress™ kinase plate and incubated overnight. On day 2, the media was gently aspirated and 100 µl of complete media with puromycin (1 µg/ml) was replaced in each well. From day 3 to day 6, fresh medium was replaced as necessary, and wells were evaluated for cell proliferation under the microscope. On day 6, the number of viable cells was determined using proliferation assay.

Proliferation assay

Proliferation was assessed using the CellTiter 96® AQueous One Solution Cell Assay (Promega, Madison, WI). Briefly, 2 x 10³ cells/well were plated in 96-well plates in culture medium (RPMI 1640 supplemented with 10% fetal bovine serum and penicillin/streptomycin) and incubated at 37°C with 5% CO₂ for a period as described above. After culture, 20 µl of CellTiter 96® AQueous One Solution Reagent was added to each well and the plates were incubated for 4 h. The absorbance was measured at 490 nm on a SPECTRAMax® Microplate Spectrophotometer (Molecular Devices, Sunnyvale, CA).

Mirk complementary DNA rescue assay

The lentiviral shRNA particles that are against the 3’ UTR of the target gene are included so one could rescue the knockdown phenotype with the introduction of a complementary DNA (cDNA) clone (without 3’ UTR) corresponding to the target gene. In theory, transcripts from an exogenous cDNA clone (without 3’ UTR) would not be susceptible to silencing by a shRNA that targets only the 3’ UTR of the gene compared with the endogenous copy. In order to verify the observed Mirk knockdown phenotype in osteosarcoma, Mirk lentiviral shRNA clones against the 3’ UTR was utilized in a cDNA rescue assay. Human Mirk cDNA (Genbank accession number: NM_004714) without the 3’ UTR was purchased from OriGene Technologies (Rockville, MD). Mirk cDNA was cloned into pCMV6-XL5 vector as transfection-ready DNA. Lentiviral rescue shRNA targeting the 3’ UTR of Mirk was purchased from Sigma (TRCN0000002140). First, KHOS cells were transfected with Mirk cDNA. Transfections were performed using Lipofectamine Plus reagents (Invitrogen) as follows: 5 x 10⁵ KHOS cells were plated into 90 mm tissue culture dishes and cultured overnight. Prior to transfection, the growth medium was replaced with serum free RPMI 1640 and cultured for 3 h. Lipofectamine reagent containing 5 µg of Mirk cDNA was combined with Lipofectamine Plus reagent and applied to the cells. After 4 h of culture, the media was replaced with RPMI 1640 containing 10% fetal bovine serum. At 24 h posttransfection, the Mirk transfected cells were collected and transduced with Mirk lentiviral shRNA targeting either 3’ UTR or open reading frame on a 96-well plate. At 72 h posttransduction, proliferation of cell growth was determined by CellTiter 96® AQueous One Solution Reagent as described previously (25).

Synthetic Mirk siRNA and transfection

Further confirmation of Mirk knockdown phenotype in osteosarcoma was carried out with synthetic human Mirk siRNA, which was purchased from Ambion at Applied Biosystems (Foster City, CA). The siRNA sequence targeting Mirk (Genbank accession no: NM_004714) corresponded to coding regions (sense 5’-GGACACUUCAUGUGCGGAA-3’, antisense 5’-UUCGGCAACAGA-AGUCCGc-3’) of the Mirk gene. Non-specific siRNA oligonucleotides (Dharmacon, Chicago, IL) were used as negative controls. KHOS cells were plated on 96-well plates for cell proliferation assays. Transfections were performed with siPORT™ NeoFX™ Transfection Agent (Ambion) as directed by the manufacturer. Each 96-well plate received 0.1 µg siRNA per well in a volume of 200 µl in triplicate, and each 60 mm dish received 5 µg siRNA per dish in a volume of 10 ml. Medium was replaced with RPMI 1640 supplemented with 10% fetal bovine serum 24 h after transfection.

Apoptosis assay

Quantification of apoptosis was evaluated using the M30-Apoptosense® ELISA assay kit as per manufacturer’s instructions (Peviva AB, Bromma, Sweden). The M30-Apoptosense ELISA measures the levels of soluble cytoskeleton 18 fragments containing the cytokeratin 18-Asp396 neoepitope, KHOS, U-2OS, Saos or normal osteoblast cell lines transduced with Mirk lentiviral shRNA or transfected with synthetic Mirk siRNA were seeded at 8000 cells/well in a 96-well plate for 24 h. After incubation, the cells were then lysed by adding 10 µl of 10% NP-40 per well, and the manufacturer’s instructions for the apoptosis assay were then followed.

Western blotting

Protein lysates from osteosarcoma tissues and cells were harvested for lysis with 1 x RIPA Lysis Buffer (Upstate Biotechnology, Charlottesville, VA). The protein concentrations were determined using Protein Assay Reagents (Bio-Rad, Hercules, CA) and spectrophotometer quantification (Beckman DU-6400). Twenty-five micrograms of total protein was processed on Nu-Page 4-12% Bis-Tris Gel (Invitrogen) and transferred to a pure nitrocellulose membrane (Bio-Rad). The rabbit polyclonal antibody to human Mirk was kindly provided by Dr Eileen Friedman from State University of New York, Syracuse, New York. Antibodies directed against actin were obtained from Santa Cruz Biotechnologies (Santa Cruz, CA). Primary antibodies were incubated at a 1:1000 dilution in Tris-buffered saline, pH 7.4, with 0.1% Tween 20 and overnight at 4°C. Signal was generated through incubation with horseradish peroxidase-conjugated secondary antibodies (Bio-Rad) in Tris-buffered saline, pH 7.4, with 5% non-fat milk and 0.1% Tween 20 at 1:2000 dilution for 1 h at room temperature. Positive immunoreactions were detected by using SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL).

Osteosarcoma tissue microarray and immunohistochemistry

The osteosarcoma tissue microarray was purchased from Imgenex Corp (San Diego, CA), which contains 58 samples from 58 osteosarcoma patients. Slides of 5 mm sections of the array were baked at 60°C for 2 h, deparaffinized in xylene for 10 min, transferred through 100% ethanol for 5 min and then rehydrated with decreasing grades of ethanol. Endogenous peroxidase was quenched by a 10 min incubation in 3% hydrogen peroxide in methanol. Antigen retrieval was performed with Target Retrieval Solution (Vector Laboratories, Burlingame, CA) following the instruction of the manufacturer. After antigen retrieval, the slides were washed with phosphate-buffered saline (parathyroid hormone, 7.5) three times at room temperature. Protein was blocked by incubating the slides in 5% normal goat serum and 1% bovine serum albumin in phosphate-buffered saline for 1 h. Primary Mirk antibody (Sigma) was applied at 4°C overnight (1:100 dilution) in 1% bovine serum albumin with 5% normal goat serum. After three 2 min rinses in phosphate-buffered saline, bound antibody was detected with the Vectastain ABC kit (Vector Laboratories) and visualized with 3,3’-diaminobenzidine high-sensitivity substrate from Vector Laboratories. Finally, the slides were counterstained with hematoxylin QS (Xantus) mounted (Vector Laboratories) and mounted with VectaMount AQ (Vector Laboratories) for long-term preservation. Mirk positive samples were defined as those showing nuclear and cytoplasmic staining pattern of tumor tissue. Light microscopic images were documented using a Nikon Eclipse Ti-U fluorescence microscope (Nikon Corp) with an attached SPOT RT digital camera (Diagnostic Instruments, Sterling Heights, MI).

Data analysis

Values shown are representative of duplicate determinations in two or more experiments. The correlation between Mirk expression levels and prognosis was analyzed by Kaplan–Meier survival analysis (GraphPad PRISM® 4 software; GraphPad Software, San Diego, CA). A two-sided Student’s t-test (GraphPad PRISM® 4 software; GraphPad Software, San Diego, CA) was used to compare the Mirk intensity scores among survivors and non-survivors. The effects of Mirk knockdown in osteosarcoma cells were also evaluated using the two-sided Student’s t-test (GraphPad PRISM® 4 software; GraphPad Software, San Diego, CA). Errors are standard deviation of averaged results and P values <0.05 were accepted as a significant difference between means.

Results

Mirk is a new regulator of osteosarcoma cell survival and apoptosis

To investigate the potential function of kinases in the proliferation and survival of osteosarcoma cells, we used a lentiviral shRNA kinase library to screen the osteosarcoma cell line KHOS. This library contains 3109 lentiviruses carrying shRNA sequences targeting 673 human kinase genes, allowing for quick high throughput loss-of-function screens. Among the 673 targeted kinase genes, we found that knocking down the expression level of four kinases led to inhibitory growth effects (unpublished data). These kinases include Mirk, PLK1, ROCK1 and PITSLRE. Since Mirk kinase has been reported to be highly expressed in several types of cancer cells, but had no biological characterization in osteosarcoma, it was selected for further study. Lentiviral shRNA targeting Mirk, when transduced into KHOS cells, led to significantly reduced cell proliferation and eventually cell death (Figure 1A). Lentiviral shRNA clones targeting the 3’ UTR of Mirk...
were utilized along with a Mirk open reading frame in a cDNA rescue assay to confirm that the Mirk knockdown phenotype in osteosarcoma was not caused by off-target effects. Transcripts from an exogenous cDNA clone lacking a 3’ UTR would not be susceptible to silencing compared with the endogenous copy. With the introduction of Mirk cDNA (without the 3’ UTR), the clone rescued the knockdown phenotype induced by lentiviral shRNA targeting of the 3’ UTR region in Mirk (Figure 1B). Western blot analysis confirmed restored Mirk expression in Mirk cDNA transfected and lentiviral shRNA rescue particles infected cells (Figure 1C). In addition to Mirk (Dyrk1b), four additional mammalian Dyrk-related kinases have been identified: Dyrk1A, Dyrk2, Dyrk3 and Dyrk4. The MISSION®/C210 LentiExpress™ Human Kinases shRNA library used in this study covers all five of these kinases. The results showed that only Mirk (Dyrk1b) knockdown significantly decreases osteosarcoma cell proliferation and induces apoptosis (Figure 1). Thus, specific lentiviral shRNA against Mirk has no effect on other isoforms of the Dyrk family (unpublished data). Similar observations have been found in other types of human tumor cells by previous studies (26–30).

Mirk is highly expressed in sarcoma cell lines and in osteosarcoma tissues

In order to examine the expression of Mirk in other tumor cell lines, osteosarcoma (U-2OS, KHOS), uterine sarcoma (MES-SA), chondrosarcoma (CS-1) synovial sarcoma (SS-1), Ewing sarcoma (TC-71) and ovarian cancer (SKOV-3, 3A, 2008) were evaluated by western blot. High levels of Mirk expression were visible in all tumor cell lines (Figure 2A). However, normal human osteoblast cell lines HOB-c, NHost and hFOB expressed very low levels of Mirk (Figure 2A). Six freshly isolated primary osteosarcoma specimens were also examined to evaluate the possibility that Mirk expression is an artifact induced by in vitro propagation. High-level expression of Mirk was observed in five of the six osteosarcoma patient samples (Figure 2B).

Mirk knockdown decreases cell proliferation and induces apoptosis in multiple osteosarcoma cells

To examine whether shRNA-mediated knockdown of Mirk causes growth inhibition and apoptosis in a wider array of osteosarcoma cells, apoptosis and cell growth assays were extended to additional osteosarcoma cell lines. The additional cell lines included U-2OS, Saos and a primary osteosarcoma in culture, OSA344. The effect of Mirk siRNA was examined on a benign human osteoblast cell line to confirm that Mirk shRNA is specific to malignant osteoblasts. Mirk siRNA knockdown significantly decreased cell proliferation in several osteosarcoma cell lines, including U-2OS (25%), Saos (35%) and OSA344 (42%) (Figure 3). However, Mirk shRNA knockdown did not have any influence on the proliferation of the benign osteoblast cell line HOB-c (Figure 3).

Mirk knockdown induces apoptosis by synthetic siRNA

Although it is highly unlikely that the same phenotype would be seen in cells transduced with multiple shRNAs targeting different regions of the Mirk gene due to some sort of lack of specificity, we chose to confirm the results by utilizing chemically synthetic siRNA. The siRNA oligonucleotide has been validated in Hela cell line siPORT™ NeoFX™ Transfection Agent for the inhibition of Mirk expression (http://www5.appliedbiosystems.com/tools/sirna/). After siRNA targeting Mirk or non-specific siRNA oligonucleotide, controls were transfected into KHOS, proliferation of the osteosarcoma cells and Mirk protein expression were measured. Although transfection with non-specific siRNA did not affect the growth rate of the cells, transfection with Mirk-targeting siRNA significantly inhibited cell proliferation (Figure 4A). Transfection with non-specific siRNA did not
show any effect on apoptosis, whereas Mirk-targeting siRNA induced strong levels of apoptosis (Figure 4B). Western blot showed siRNA Mirk knockdown reduces Mirk protein expression (Figure 4C).

**Mirk expression levels correlates with clinical prognosis in osteosarcoma patients**

Mirk expression was analyzed by immunohistochemistry using an osteosarcoma tissue microarray. Immunohistochemical analyses determined that all tumors present on the tissue microarray had positive staining for Mirk. Mirk staining intensity was graded into three groups: low staining (1+), moderate staining (2+) and high staining (3+). Of the 58 patients examined, 22 (37.9%) patients were classified as low staining, 23 (39.7%) patients as moderate staining and 13 (22.4%) as high staining. Average follow-up periods for patients in the low three staining groups were 76.6, 70.8 and 28.5 months, respectively. When comparing the clinical characteristics of low-staining, moderate-staining and high-staining osteosarcoma, no correlation existed between Mirk expression and age, gender or tumor location ($P > 0.05$) (Table I). Kaplan–Meier survival analysis showed that the prognosis for patients in the Mirk high-staining group was significantly worse than those in the Mirk low-staining and moderate-staining groups ($P = 0.001$) (Figure 5). Patients were also separated into two groups according to the 60 months survival rate: survivors (survived up to 60 months after follow-up) and non-survivors (deceased within 60 follow-up months). Mirk staining intensity was compared between samples from survivors and non-survivors. Thirty-two (55.2%) samples from survivors and 26 (44.8%) samples from non-survivors were collected. The level of Mirk staining for samples from non-survivors were significantly higher than that of survivors ($P = 0.0012$), where the average Mirk expression levels for survivors and non-survivors were 1.5–2.2, respectively (Figure 5).

**Discussion**

We performed a human kinase shRNA screen and identified essential kinases based on their requirement for growth and survival of osteosarcoma cells. This study provides a genomic view on the role of kinases in the regulation of osteosarcoma cell survival and death. Our study shows that decreased Mirk expression resulted in decreased cell proliferation and increased cell death and therefore may indicate a previously unrecognized role for Mirk in osteosarcoma. Most importantly, normal osteoblast cells seem to be less sensitive to Mirk expression levels correlates with clinical prognosis in osteosarcoma patients.

![Fig. 3.](image-url) Knocked down Mirk expression decreases cell proliferation in multiple osteosarcoma cell lines. (A) Cell proliferation was determined by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-di-phenyltetrazolium bromide on cell lines: U-2OS, Saos, OSA344 and normal osteoblast cell HOB-c, after transduction with Mirk shRNA (NM_004714.x-442s1c1). The absorbance values were normalized by the control cells (non-infected) to 100% survival and the values of Mirk shRNA transduced cells were then quantified to get relative percentage of cell survival. Mirk knockdown decreases cell proliferation in osteosarcoma cells but not in normal osteoblast cells. (B) Phase contrast photomicrographs of cell lines before and after infection of lentiviral Mirk shRNA. Mirk knockdown induces significantly cell death in osteosarcoma cell lines U-2OS, Saos and OSA344 but not in normal osteoblast cell HOB-c.
gene knockdown via RNA interference targeting, suggesting its therapeutic value in the clinic. Beyond the influence of growth and survival, the reasons for differential expression of Mirk in normal osteoblast and osteosarcoma cells remain unknown. Several reports have indicated that Mirk mediates the survival of colon carcinoma cells and is implicated in clonogenicity of rhabdomyosarcoma, pancreatic cancer and lung cancer cell lines (26,30–32). The kinase Mirk is a member of a conserved family of serine/threonine kinases, which are activated by intramolecular tyrosine phosphorylation (27). Mirk is expressed at low levels in most normal tissues and mediates differentiation in various tissues. Mirk is upregulated and activated in skeletal muscle but is not essential for muscle formation or viability in the embryo (33,34). Other investigators have shown that Mirk has antiapoptotic functions in cancer cells in which it is highly expressed and activated, such as in pancreatic cancer cells, rhabdomyosarcoma cells, some colon carcinoma cells and HeLa cervical carcinoma cells (13,26,31,32). Here, we also show that osteosarcoma cells have a high level of Mirk expression when compared with normal osteoblast cells. The expression of Mirk was also confirmed in osteosarcoma tissue derived from patient samples. By targeting Mirk expression with shRNA or siRNA, osteosarcoma cell growth could be inhibited, inducing apoptosis among various cell lines. However, knockdown of Mirk in benign osteoblast cells was

Fig. 4. Synthetic siRNA targeting Mirk decreases cell proliferation and induces apoptosis in KHOS cells. (A) KHOS cells were transfected with Mirk siRNA or non-specific siRNA, and cell proliferation posttransfection was determined by CellTiter 96® AQueous One Solution Reagent. (B) Apoptosis was evaluated using the M30-Apoptosense ELISA assay kit. The increase levels of cytokeratin 18 reflect Mirk siRNA induced apoptosis in KHOS transfected cells. (C) Synthetic siRNA Mirk knockdown reduces Mirk protein expression as determined by western blot.

Table I. Comparison of the clinical characteristics of 58 patients with Mirk staining

<table>
<thead>
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<th>Characteristic</th>
<th>DyRK 1B</th>
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<tr>
<td></td>
<td>Low staining</td>
<td>Medium staining</td>
<td>High staining</td>
<td>P</td>
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<tr>
<td>Age at diagnosis (years)</td>
<td>16.7 25.1 21</td>
<td>11–27 9–59 5–61</td>
<td>&gt;0.05</td>
<td></td>
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<tr>
<td>Gender (%)</td>
<td>Male 15 (68.2) 17 (73.9) 9 (69.2)</td>
<td>Female 7 (31.8) 6 (26.1) 4 (30.8)</td>
<td>&gt;0.05</td>
<td></td>
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<tr>
<td>Location (%)</td>
<td>Axial 1 (4.5) 5 (21.7) 2 (15.4)</td>
<td>Extremities 21 (95.5) 18 (78.3) 11 (84.6)</td>
<td>&gt;0.05</td>
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<td>Prognosis</td>
<td>Survival 14 (63.6) 12 (52.2) 1 (7.7)</td>
<td>Non-survival 8 (36.4) 11 (47.8) 12 (92.3)</td>
<td>&lt;0.05</td>
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*a = 22.
*b = 23.
*c = 13.
not effective in inducing growth arrest, implying that Mirk does hold a role in promoting the integrity and proliferation of malignant osteoblast cells. The mechanisms of Mirk signaling still remain incompletely understood (27), but evidence supports an important role for Mirk in osteosarcoma cells, suggesting that Mirk may be pursued as a novel strategy in the treatment of osteosarcoma.

Although upregulation of Mirk expression and/or constitutive activation of this kinase have been observed in several different types of cancer, the relationship between its expression and the clinical characteristics of patients was not previously investigated. We analyzed the immunohistochemical staining of Mirk expression in tissue samples from 58 patients with osteosarcoma and analyzed the relationship between its expression with clinical characteristics and outcomes. In our study, we found that a high-staining level of Mirk was detected in 22.4% of tumor samples examined. The data indicate that changes in Mirk expression level are frequent in osteosarcoma. However, the level of Mirk expression did not correlate with age, gender or location. On the other hand, with respect to osteosarcoma patients, the data indicate that the overall survival rate of patients with tumors that express high levels of Mirk was significantly shorter than those with low or moderate levels. The levels of Mirk staining in samples from patients that deceased within 60 follow-up months were significantly higher than those who survived at 60 follow-up months (P = 0.0012). We show for the first time that Mirk is an independent prognostic factor in osteosarcoma.

In summary, the present study demonstrates that the kinase Mirk is essential for the growth and survival of osteosarcoma cells. The high expression of Mirk in the lesions serves as a biomarker for prognosis. In addition, knockdown of Mirk expression leads to apoptosis in multiple osteosarcoma cell lines and inhibiting cellular proliferation in vitro. This highlights the potential for Mirk to serve as a possible target for molecular therapy in the treatment of osteosarcoma.

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**References**


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