MMP-2 regulates Src activation via repression of the CHK/MATK tumor suppressor in Osteosarcoma

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Abstract

Doxorubicin, a first-line anticancer drug for osteosarcoma treatment, has been the subject of recent research exploring the mechanisms behind its chemoresistance and its ability to enhance cell migration at sublethal concentrations. Matrix metalloproteinase-2 (MMP-2), a type IV collagenase and zinc-dependent endopeptidase, is well-known for degrading the extracellular matrix and promoting cancer metastasis. Our previous work demonstrated that nuclear MMP-2 regulates ribosomal RNA transcription via histone clipping, thereby controlling gene expression. Additionally, MMP-2 activity is regulated by the non-receptor tyrosine kinase and oncogene, Src, which plays a crucial role in cell adhesion, invasion, and metastasis. Src kinase is primarily regulated by two endogenous inhibitors: C-terminal Src kinase (Csk) and Csk homologous kinase (CHK/MATK). In this study, we reveal that the MMP-2 gene acts as an upstream regulator of Src kinase activity by suppressing its endogenous inhibitor, CHK/MATK, in osteosarcoma cells. We also show that enhanced osteosarcoma cell migration which is induced by sublethal concentrations of doxorubicin can be overcome by inactivating the MMP-2 gene or overexpressing CHK/MATK. Our findings highlight the MMP-2 gene as a promising additional target for combating cancer cell migration and metastasis. This is due to its impact on the gene and protein expression of the tumor suppressor CHK/MATK in osteosarcoma. By targeting the MMP-2 gene, we can potentially enhance the effectiveness of doxorubicin treatment and reduce chemoresistance in osteosarcoma.

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Abstract

Doxorubicin, a first-line anticancer drug for osteosarcoma treatment, has been the subject of recent research exploring the mechanisms behind its chemoresistance and its ability to enhance cell migration at sublethal concentrations. Matrix metalloproteinase-2 (MMP-2), a type IV collagenase and zinc-dependent endopeptidase, is well-known for degrading the extracellular matrix and promoting cancer metastasis. Our previous work demonstrated that nuclear MMP-2 regulates ribosomal RNA transcription via histone clipping, thereby controlling gene expression. Additionally, MMP-2 activity is regulated by the non-receptor tyrosine kinase and oncogene, Src, which plays a crucial role in cell adhesion, invasion, and metastasis. Src kinase is primarily regulated by two endogenous inhibitors: C-terminal Src kinase (Csk) and Csk homologous kinase (CHK/MATK). In this study, we reveal that the MMP-2 gene acts as an upstream regulator of Src kinase activity by suppressing its endogenous inhibitor, CHK/MATK, in osteosarcoma cells. We also show that enhanced osteosarcoma cell migration which is induced by sublethal concentrations of doxorubicin can be overcome by inactivating the MMP-2 gene or overexpressing CHK/MATK. Our findings highlight the MMP-2 gene as a promising additional target for combating cancer cell migration and metastasis. This is due to its impact on the gene and protein expression of the tumor suppressor CHK/MATK in osteosarcoma. By targeting the MMP-2 gene, we can potentially enhance the effectiveness of doxorubicin treatment and reduce chemoresistance in osteosarcoma.

Keywords

Matrix metalloproteinase-2 (MMP-2); Csk homologous kinase (CHK/MATK); Src Family Kinases (SFK); Src; Doxorubicin; Osteosarcoma

Introduction

Matrix metalloproteinases (MMPs) constitute a family of zinc-dependent endopeptidases overexpressed in several cancer types (1,2). In particular, matrix metalloproteinase-2 (MMP-2) gained considerable attention for its ability to degrade the extracellular matrix, facilitating detachment from primary tumors and migration to secondary tumor sites, thereby underscoring its critical role in cancer metastasis (3). Currently MMP inhibitors employed for cancer treatment predominately target extracellular MMPs, but the lack of specificity (broad targeting of multiple MMPs) renders them less effective in impeding cancer metastasis due to dose-limiting toxicity (4,5). Recent findings reveal that MMP-2 also localizes to different subcellular compartments, including the nuclei of osteosarcoma U2OS cells. However, the contribution of intracellular MMP-2 in osteosarcoma cell migration remains largely unexplored (6,7).

Previous research demonstrated that MMP-2 expression is regulated downstream of Src activity, a non-receptor tyrosine kinase and oncogene, through the extracellular signal-regulated kinases (ERK) pathway (8–11). Src kinase, a member of Src Family Kinases (SFKs), is overexpressed in cancer, and serves a critical role in cell adhesion, invasion and cancer metastasis (9). Src's catalytic activity is regulated via phosphorylation at Tyr-416 for full activation, and Tyr-527 for inhibition (12). Although phosphorylation at Tyr-527 is indicative of Src inhibition, SFKs' receptor protein-tyrosine kinases may non-catalytically bind to the SH2 and SH3 domains to inhibit the Src kinase (13,14). The overactivation of SFKs, such as Src, can be attributed in part to the reduced expression of their endogenous inhibitors (13).

The most common endogenous inhibitors of SFKs include C-terminal Src kinase (Csk) and the Csk homologous kinase (CHK/MATK) (14). While Csk is ubiquitously expressed in mammalian cells, CHK/MATK is predominantly found in hematopoietic cells and neurons (14–16). Csk and CHK/MATK share a similar structural composition with Src, possessing a SH2, SH3 and kinase domain; however, they lack the C-terminal

tail phosphorylation site and N-terminal myristoyl group (14). Despite their structural resemblance, the binding domains of Csk and CHK/MATK exhibit differences, as their SH2 domains engage with distinct phosphoproteins and target Csk and CHK/MATK to various cellular compartments (17). Both inhibitors were previously reported to catalyze the phosphorylation of the C-terminal tail tyrosine of Src at Tyr-527, but recent studies have shown CHK/MATK to be ineffective at phosphorylating Src C-terminal regulatory Tyr-527 (13). Unlike Csk, CHK/MATK has also been shown to directly bind to Src via a non-catalytic mechanism, thereby preventing autophosphorylation at Tyr-416 and inhibiting Src activation without affecting Tyr-527 phosphorylation, and subsequently, inhibit cellular processes such as cell migration (18,19).

Doxorubicin, an anthracycline antibiotic, is commonly used to treat various cancer types, including osteosarcoma, breast cancer and leukemia (20). Specifically, in osteosarcoma, it serves as a first line drug treatment; however, low concentrations result in drug resistance, while at high concentrations cause significant toxicity (21,22). Due to doxorubicin's toxic effects on the heart, brain, liver and kidneys, doxorubicin doses need to be lowered in various clinical settings and research has increasingly focused on the cellular mechanisms influenced by different concentrations of doxorubicin (20). For instance, a study by Mohammed *et al.* (2021) investigated the impact of a sublethal concentration of doxorubicin on several cancer cell lines, revealing that sublethal concentrations enhances cell migration and invasion through SFK activation in both non-invasive and invasive cancer cell lines, including U2OS (23). Furthermore, doxorubicin has been reported to increase the expression of MMP-2 and MMP-9 in cardiac myocytes (24,25). These findings align with the study by Mohammed *et al.* (2021), as a sublethal concentration of doxorubicin activates SFKs, augmenting the expression of MMP-2, and consequently, enhancing cell migration (23).

The role of intracellular MMP-2 is increasingly being implicated not only in cancer cell invasion, but also in cell migration (6). In our previous studies, we reported that nuclear MMP-2 regulates ribosomal RNA transcription through histone clipping, thereby modulating gene expression and cell proliferation (7). This discovery has opened up a new avenue of research on the role of intracellular/nuclear MMP-2 in regulating gene expression, as cleavage of histones will lead to modified chromatin structure and epigenetic alterations regulating gene expression. In the current study, we examined the impact of sublethal concentrations of doxorubicin on enhancing the invasiveness and migration of U2OS cells in the absence of MMP-2 gene. We reported that knocking out of MMP-2 gene considerably hinders osteosarcoma cell migration and inhibits doxorubicin-induced cell migration. Additionally, we found that the MMP-2 gene plays a role in regulating Src activation, and consequently, cell migration. We also report that inactivation of MMP-2 inhibits Src activation through upregulating the endogenous Src inhibitor, CHK/MATK. Lastly, although a sublethal concentration of doxorubicin promotes osteosarcoma cell migration, combining this treatment with CHK/MATK overexpression in osteosarcoma cells hinders, or at least partially attenuates, cell migration. We conclude that a deeper understanding of the role of intracellular/nuclear MMP-2 in cell migration may pave the way for new strategies to effectively target cancer migration and metastasis.

Materials and Methods

Antibodies and reagents

The reagents and antibodies purchased include: Dulbecco's modified Eagle's medium (DMEM), Corning; fetal bovine serum (FBS), Krackler; Src (2109T), phospho-Src Family (Tyr416) (6943T), phospho-Src (Tyr527) (2107T), CHK/MATK (20729S), Csk (4980T), beta-Actin (4970T), GAPDH (97166T), Cell Signaling Technology; MMP-2 (ab92536), Abcam; Doxorubicin hydrochloride (D1515-916), Sigma; Lenti ORF particles, MATK (mGFP-tagged)- Human megakaryocyte-associated tyrosine kinase (MATK) transcript variant 1, Origene.

Cell Culture

Human osteosarcoma cell line (U2OS; ATCC-HTB-96) were cultured at 37 in a humidified 5% CO_2 atmosphere in DMEM supplemented with 10% FBS. U2OS WT+ GFP-MATK stables were additionally supplemented and cultured with 4 μg/mL puromycin. MMP-2 knockout U2OS cells were generated by CRISPR/Cas-9, as previously described (7).

Lentiviral Transduction

U2OS WT cells were seeded at 300,000 cells in a 35 mm dish (50% confluency) and cultured for 24 hours under standard culture conditions. The number of viral particles were calculated according to the multiplicity of infection (MOI), 5 MOI per U2OS cell, totaling 1.5 x 10^6 total transducing units needed. The appropriate number of lentiviral particles (MATK-GFP-tagged), culture medium, and 4 µg/mL polybrene to the total volume of 500 µL was added to the 35 mm dish and cultured overnight. The next day after transduction, the medium containing lentiviral particles was removed and replaced with fresh medium. 72 hours after transduction, a stable cell line was generated using 4 µg/mL of puromycin (drug resistant marker).

qPCR

U2OS cells were seeded on 10 cm plates under standard culture conditions and incubated for 24-48 hours to reach 100% confluency. Once cells reached confluency, RNA from cells were isolated using RNeasy Plus Mini Kit (74134, Qiagen). Using the RNA, cDNA was synthesized using qScript cDNA SuperMix and protocol from Quantabio (101414-102). The qPCR primer cocktails were made using PerfeCTa SYBR Green FastMix (101414-276), forward primer, reverse primer (Real Time Primers) and RNase/DNase free H_2O. In a 96 well qPCR plate, 18 µl of primer cocktail was added and 2 µl of cDNA, accordingly. qPCR was run using BioRad cfx96 and fold gene expression was calculated.

Western blotting

Cell lysis samples were run through gel electrophoresis using 10% SDS-PAGE gels and proteins were electrotransfered onto PVDF membranes (97062-900, VWR). The membranes were then blocked with 5% dried skimmed milk in TBS-T (50 mM Tris pH 8.4, 0.9% NaCl, 0.05% Tween-20) for 1 hour and incubated overnight at 4°C in the presence of previously listed primary antibodies in 5% milk. Prior to incubating the membrane in the secondary antibody for 1 hour at 25°C, it was washed with TBS-T 3 times for 10 min. each. The membrane was developed using Radiance Plus Reagents (AC2103) and chemiluminescent bands were revealed (Azure Biosystems).

Gelatin Zymography

Cell lysis samples (20 ug of protein per sample) were prepared with 4x Laemmli buffer without 2-mercaptoethanol and samples were not boiled. Samples were electrophoresed through 8% tris-glycine polyacrylamide gel with 0.1% gelatin and gels were washed with 2.5% Triton X-100 3 times for 20 minutes each. Gels were incubated in an incubation buffer (NaCl, $CaCl_2$, Tris base) overnight at 37°C. The next day, the gel was stained with 0.05% Coomassie Brilliant Blue G-250 stain (Sigma B1131) for 1 hour and destained with a destaining solution (methanol, glacial acetic acid, ddH_2O) overnight at 25°C. Bands were visualized (Azure Biosystems).

Cytotoxicity assay

In a 96 well plate, 5,000 U2OS WT cells were seeded and cultured for 24 hours under standard culture conditions. Treated cells with various concentrations of doxorubicin hydrochloride for 24 and 48 hours. After 24 and 48 h, LDH activity was measured using reagents from CyQUANT LDH Cytotoxicity Assay Kit (C20300, invitrogen). Absorbance was read at 490 nm and 680 nm to determine LDH activity. Percent cytotoxicity was calculated using the following: [(Compound-treated LDH activity-Spontaneous LDH activity)/(Maximum LDH activity-Spontaneous LDH activity)] *100.

Transwell migration assay

In 24 well plates with transwell chamber inserts (Costar 3464, Corning), U2OS WT and MMP-2 KO cells were seeded, separately, in the upper chamber at 20,000 cells in 200 µl serum-free DMEM. In the bottom chamber of the well 800 µl DMEM supplemented with 10% FBS was added. The plates containing triplicates of WT and KO were cultured for 24 hours under standard culture conditions. The following day, non-migrating cells were removed from the upper chamber and migrating cells were fixed with 70% ethanol and stained with 0.2% crystal violet. Membranes were left to dry and images were taken.

Wound closure assay

In 6 well plates, U2OS WT, MMP-2 KO and GFP-MATK stable cells were cultured, separately, for 24 hours under standard culture conditions to reach 100% confluency. Once confluent, a vertical scratch was formed down the middle of the well using a 200 µl pipette tip. Cells were washed with 1x PBS and OPTI-MEM was added to each well. The appropriate concentrations of doxorubicin were added to the OPTI-MEM in respective wells. Images of the wound were taken at 0 hour, 24 hour and 48 hour time points. The percent wound closure at 24 and 48 h were calculated as the following: [(Initial wound width-final wound width)/(initial wound width) *100].

Statistical analysis

The results of this study are expressed as mean ± standard error of the mean. The statistical significance between the mean values were analyzed by Student's t-test using GraphPad Prism 9. Significant differences; $p < 0.05$.

Results

MMP-2 Knockout Impairs Cell Migration in Osteosarcoma

To validate our U2OS MMP-2 knockout cell line, we analyzed the mRNA levels and examined the fold change of MMP-2 gene expression between the wildtype (WT) and MMP-2 knockout (KO). qPCR analysis of MMP-2 mRNA showed a significant difference between the KO and WT, with the KO displaying an 833-fold decrease in MMP-2 transcripts compared to the WT cells (Figure 1a). The MMP-2 gene inactivation in KO was also confirmed at the protein level by performing western blot and analyzing the activity of MMP-2 in our WT and KO cells by gelatin zymography (Figure 1a).

In order to assess the impact of MMP-2 KO on cancer cell migration, transwell migration assays were conducted on U2OS WT and KO cell lines. Our findings demonstrated that while the WT cells displayed a high level of migration ability, the MMP-2 KO cells exhibited complete inhibition of cell migration (Figure 1b). Interestingly, adding active MMP-2 enzymes exogenously to the serum-free media of KO cells did not restore cell migration. Likewise, adding the culture media from WT cells, that contains active secreted MMP-2, to the KO cells did not enhance cell migration. It is noteworthy that although prior research had established that MMP-2 inhibitors results in inhibition of tumor invasion and angiogenesis attributable to the activity of extracellular MMP-2 (26), our study is the first to demonstrate a significant inhibition in osteosarcoma cell migration as a result of inactivating MMP-2 gene in the presence of active extracellular MMP-2.

To further examine the effect of MMP-2 KO on the migratory ability of osteosarcoma cells, we conducted wound closure assays on both the WT and MMP-2 KO cells for the duration of 48 hours until complete wound closure was observed in the WT (Figure 1c). Minimal wound closure (approximately 35% closure vs. 100% closure in WT) was observed in the MMP-2 KO at 48 hours time-point (Figure 1c). Thus, knocking out the MMP-2 gene in osteosarcoma cells, in the presence of active extracellular MMP-2, results in a significant inhibition of cancer cell migration, suggesting that the MMP-2 gene, rather than extracellular protein, plays a major role in cancer cell migration pathways.

Sublethal concentrations of Doxorubicin enhance osteosarcoma cell migration in an MMP-2-dependent manner

Previous research by Mohammed *et al.* (2021) revealed that sublethal concentrations of doxorubicin enhances cell migration in several cancer cell lines, including U2OS (23). To determine the concentrations of doxorubicin causing a sublethal effect on our U2OS cell line, a LDH release assay was performed. The highest sublethal concentration of doxorubicin on U2OS was 0.4 µM for both 24- and 48-hour treatments (Figure 2a). This doxorubicin sublethal concentration enhanced cell migration in WT cells using wound closure assays; albeit statistically non-significant, with nearly complete wound closure (approximately more than 80% closure) observed at 24 hours, compared to approximately 60% wound closure in the untreated sample

(Figure 2b), supporting a previous study (23). Despite these results in the WT, the sublethal concentration of doxorubicin failed to enhance cell migration in MMP-2 KO cells, which continued to show minimal migration at 24 and 48 hours without or with 0.4 μM doxorubicin treatment (25% vs. 20%, respectively) (Figure 2c).

Our findings indicate that sublethal concentrations of doxorubicin augment cell migration in WT U2OS cells. However, this increase in cell migration is negated when the MMP-2 gene is knocked out. This impairment in cell migration in MMP-2 KO cells provides further evidence of the MMP-2 gene's involvement in cancer cell migratory pathways.

MMP-2 gene mediates doxorubicin-induced phosphorylation of Src at Tyr-416

Mohammad *et al.* (2021) showed that increases in Src phosphorylation at Tyr-416, representing active Src, occurs when cancer cells are treated with sublethal concentrations of doxorubicin (23). The increase in phosphorylated Src at Tyr-416 serves as an indicator of Src activation, which plays an important role in cytoskeletal reorganization and cell migration (12,27). We sought to investigate Src activation induced by sublethal concentrations of doxorubicin in both WT and MMP-2-KO cells. As a result, the WT and MMP-2 KO cells were treated with 0.4 μM doxorubicin for 24 hours and pSrc at Tyr-416 was measured (Figure 3a). Western blots show Src phosphorylation at Tyr-416 was indeed significantly increased when WT cells were treated with a sublethal concentration of doxorubicin. On the other hand, increased Src phosphorylation at Tyr-416 was disrupted in MMP-2 KO cells when treated with the same doxorubicin concentration (Figure 3b).

Previous research also revealed that increases in Src phosphorylation at Tyr-527 are observed in cases when Src is inhibited by specific upstream kinases (28). To determine whether Src at Tyr-527 was phosphorylated in the WT or MMP-2 KO cells, the levels in both untreated and 0.4 μM doxorubicin treated cells were examined (Figure 3a, b). Western blots and corresponding quantifications show non-significant change in phosphorylation of Src at Tyr-527 among the WT and MMP-2 KO, either untreated or treated with doxorubicin (Figure 3a, b). Therefore, the results suggest the disruption of Src phosphorylation at Tyr-416 in MMP-2 KO cells is not as a result of increased phosphorylation at Tyr-527 residue. However, our results indicate that MMP-2 gene is an upstream mediator of a signaling pathway that regulates doxorubicin-induced activation and phosphorylation of Src at Tyr-416 in osteosarcoma cells.

MMP-2 downregulates expression of Src Family kinase inhibitors

To elucidate the mechanism underlying the lack of Src phosphorylation at Tyr-416 in MMP-2 KO cells, we analyzed the expression and activity of upstream endogenous SFK inhibitors at both the RNA and protein levels. Based on current literature, we examined three important regulators of the Src Family Kinases: CHK/MATK, Csk and CDC2 (13,29,30). The qPCR showed a significant upregulation of the CHK/MATK gene expression in the MMP-2 KO cells compared to WT cells (110-fold increase) (Figure 4a). Both CDC2 and Csk transcripts were also slightly upregulated in the MMP-2 KO cells (7.0-fold and 2.1-fold, respectively). Nonetheless, our attention was drawn to the substantial increase in CHK/MATK expression within the MMP-2 KO cells (Figure 4a).

Due to the significance of both SFK inhibitors, CSK and CHK/MATK, protein levels were measured, as shown in the western blot in Figure 4b. CSK protein levels were not significantly different between the WT and MMP-2 KO conditions in either untreated or 0.4 μM doxorubicin-treated cells. CHK/MATK protein levels, however, were only detected in the MMP-2 KO cells and were significantly upregulated compared to the WT cells in both doxorubicin-treated and untreated cells (Figure 4b). The increased expression of CHK/MATK, a SFK inhibitor, in MMP-2 KO cells may explain the lack of Src phosphorylation/activation by doxorubicin in these cells. This also suggests that MMP-2 regulates the expression of CHK/MATK gene in osteosarcoma.

CHK/MATK overexpression inhibits osteosarcoma cell migration induced by sublethal concentration of doxorubicin

To determine the effect of overexpressing CHK/MATK in osteosarcoma, we transduced WT cells with GFP-MATK lentiviral particles and created a stable cell line. WT+MATK stable cell line was validated with qPCR and western blot (Figure 5a). After validation, we conducted wound closure assays on U2OS WT and WT+MATK stable cells with and without 0.4 μM doxorubicin treatment for 48 hours. The untreated wound closure assay did not show a significant difference in cell migration between U2OS WT and WT+MATK stable cell lines as these CHK/MATK cells were able to migrate (Figure 5b). Interestingly, however, when WT+MATK cells were treated with 0.4 μM doxorubicin, we observed a significant reduction, rather than enhancement, in cell migration, with only 30% wound closure observed (Figure 5c). Our results indicate that the overexpression of CHK/MATK in U2OS cells efficiently hinders cell migration induced by sublethal concentrations of doxorubicin.

To investigate the role of CHK/MATK in inhibiting the phosphorylation of Src at Tyr-416, we treated WT+MATK stable cells with 0.4 μM doxorubicin and examined pSrc Tyr-416 levels. Our results indicated that doxorubicin treatment caused minimal phosphorylation at Tyr-416, which was not significantly different from the untreated WT+MATK cells (Figure 5d). We also examined the phosphorylation level of Tyr-527 to determine the inhibitory mechanism of CHK/MATK in osteosarcoma. Likewise, western blot analysis showed non-significant phosphorylation at Tyr-527 with or without doxorubicin in the presence of CHK/MATK overexpression (Figure 5d). These results suggest that the inhibitory effect of CHK/MATK on Src phosphorylation on Tyr-416 is not due to an increase in phosphorylation at Tyr-527. Overall, our results demonstrate that MMP-2 knockout resulted in substantial re-expression of CHK/MATK in U2OS cells. This re-expression plays a crucial role in inhibiting cell migration induced by sublethal concentrations of doxorubicin by regulating Src phosphorylation/activation.

Discussion

Osteosarcoma, a prevalent bone cancer in adolescents, continues to exhibit high mortality rates of 30-40%, with metastasis occurring in at least 25% of patients at diagnosis (31). Although MMP-2 is known to degrade the extracellular matrix and facilitate cancer cell invasion and metastasis, targeting extracellular MMPs clinically has proven insufficient in inhibiting metastasis (3). Recent findings indicate that MMP-2 is also present in various subcellular compartments, including the nuclei of various cells such as osteosarcoma U2OS cells (7,32). We previously reported that nuclear MMP-2 can regulate gene expression of ribosomal RNA (7). Thus, examining the role of intracellular/nuclear MMP-2 in cancer cell migration pathways is crucial for developing effective osteosarcoma treatments targeting metastasis.

We here investigated the consequences of MMP-2 knockout on U2OS cell migration. We observed that wild-type (WT) cells exhibited complete wound closure within 48 hours, whereas MMP-2 knockout (KO) cells showed only 35% closure. Moreover, KO cells were unable to migrate in the presence of externally supplemented active MMP-2 within the culture media. These findings imply that the MMP-2 gene significantly hinders osteosarcoma cell migration, with extracellular MMP-2 having a minimal impact. We propose that the observed effect is attributed to the presence of intracellular/nuclear MMP-2. This protease, typically involved in the degradation of extracellular matrix components, has been found to play a role in various subcellular compartments including the nucleus (6,7). The presence of intracellular/nuclear MMP-2 in this context could potentially contribute to the enhanced cell migration observed in osteosarcoma cells treated with sublethal concentrations of doxorubicin. Further investigation is necessary to fully understand the underlying mechanisms and the role of MMP-2 in modulating osteosarcoma cell migration. Our observations align with a previous study on retinoblastoma (RB) that investigated the effects of inactivating MMP-2 or MMP-9 in cell migration, invasion, and angiogenesis (33). These studies, collectively, indicate that targeting the MMP-2 gene is crucial for impeding cancer cell migration.

At present, doxorubicin is a frequently utilized anticancer chemotherapy for treating osteosarcoma (21). Although low doxorubicin doses cause reduced cardiotoxic effects, numerous studies have reported that sublethal concentrations of doxorubicin may enhance cancer cell migration and lead to chemoresistance (23,25). Accordingly, exploring methods to boost the efficacy of this low-dose treatment in osteosarcoma cases is necessary. Huang *et al.* (2012) investigated a mechanism linked to chemoresistance, which involves the

DNA-binding protein HMGB1 (21). As one of the three anticancer drugs, doxorubicin triggers the upregulation of HMGB1 in osteosarcoma cells. This action leads to the formation of a complex with the autophagy regulator Beclin1, thereby increasing chemoresistance. Furthermore, suppressing HMGB1 effectively restored chemosensitivity in osteosarcoma cells to lower concentrations of doxorubicin (21). In a related study, Tian *et al.* (2020) observed a similar chemoresistance effect of doxorubicin on osteosarcoma cells. They discovered that doxorubicin treatment promoted stem cell-like characteristics in osteosarcoma, such as enhanced cell migration and proliferation, which ultimately resulted in resistance to the drug (34). Additionally, Tian *et al* . found that apatinib effectively deactivated the STAT3/Sox2 pathway and reduced doxorubicin-induced cell migration and chemoresistance (34). We investigated the effects of sublethal doxorubicin concentrations on osteosarcoma cells lacking the MMP-2 gene to identify an additional target for enhancing doxorubicin effectiveness. Our results show that MMP-2 knockout prevents increased cell migration in response to sublethal doxorubicin concentrations. Additionally, we demonstrated that MMP-2 gene is upstream mediator of Src kinase in the migration pathway, thus we suggest that MMP-2 expression is not only downstream of Src activation as previously reported (23).

We also found that MMP-2 regulates Src kinase activity via suppression of the endogenous Src inhibitor, CHK/MATK, in osteosarcoma cells. Cheuh*et al.* (2021) identified that CHK/MATK is suppressed and epigenetically silenced in human colorectal cancer cells, suggesting its potential role as a tumor suppressor (18). To investigate MMP-2's role in regulating the expression of endogenous Src inhibitors Csk and CHK/MATK in osteosarcoma, we analyzed gene fold expression and protein levels in U2OS WT and MMP-2 KO cells. While Csk gene fold expression was slightly elevated in MMP-2 KO cells, CHK/MATK expression was dramatically increased. Protein level analysis showed a substantial upregulation of CHK/MATK in MMP-2 KO cells, with Csk levels remaining similar between WT and KO cells. Under conditions where CHK/MATK level is elevated, achieved through MMP-2 knockout or CHK/MATK overexpression, sublethal concentrations of doxorubicin fail to activate Src kinase activity. These findings suggest that intracellular/nuclear MMP-2 is responsible for downregulating and suppressing the potential tumor suppressor CHK/MATK in osteosarcoma. Furthermore, the enhanced osteosarcoma cell migration induced by sublethal doxorubicin concentrations can be overcome by overexpressing CHK/MATK in WT cells. Thus, we propose that the MMP-2 gene is an additional target to consider, as it influences the gene and protein expression of the tumor suppressor CHK/MATK in osteosarcoma. Clinically, we anticipate that inhibiting intracellular/nuclear MMP-2 and allowing CHK/MATK re-expression will enhance the effectiveness of sublethal doxorubicin treatments in osteosarcoma patients.

In addition, we analyzed the effects of overexpressing CHK/MATK in U2OS WT cells to unveil the impact on osteosarcoma migration. Surprisingly, simply overexpressing CHK/MATK in U2OS WT cells did not reduce their migration. However, CHK/MATK overexpression was able to inhibit the doxorubicin-induced enhancement of cell migration. Previous research shows in normal untreated cells, SFKs remain in the stable inactive conformation until activated in some cellular events, including doxorubicin treatment and cell migration (17,23). We then activated the Src system through sublethal concentrations of doxorubicin, as shown in our earlier experiments, and therefore, examined Src phosphorylation at Tyr-416 and Tyr-527 in our U2OS WT cells overexpressing CHK/MATK cells. In parallel with our data showing that CHK/MATK overexpression inhibited doxorubicin-induced cell migration, there was no effect on Src activation/phosphorylation when these cells were treated with sublethal concentration of doxorubicin. Additionally, unlike Csk, CHK/MATK is also known to suppress multiple active forms of SFKs by a non-catalytic mechanism that directly binds to the C-terminal tail of SFKs. This inhibiting mechanism is independent of any Src phosphorylation that occurs at Tyr-527. Despite the lack of phosphorylation at Tyr-527, we also observed non-significant change in phosphorylation at Tyr-416 when cells overexpressing CHK/MATK were treated with doxorubicin. As a result, overexpressing CHK/MATK inhibits doxorubicin-induced Src activation and phosphorylation at Tyr-416 as well as the enhancement of cell migration in osteosarcoma.

Correspondingly, Pichot *et al.* (2009) investigated the effects of targeting SFKs with dasatinib in combination with doxorubicin treatments to inhibit the migration and invasion of breast cancer cells. A synergistic effect between dasatinib and doxorubicin treatments were observed, resulting in inhibiting cell migration, prolifer-

ation, thus significantly reducing IC_{50} of doxorubicin (35). We also report similar effects of overexpressing CHK/MATK to inhibit doxorubicin-induced Src activation and U2OS cell migration.

Conclusions

Our study, as shown in Figure 6, demonstrates that the MMP-2 gene, upstream of Src, regulates Src kinase activity by suppressing CHK/MATK, the endogenous Src inhibitor, in osteosarcoma cells. Furthermore, the enhanced cell migration caused by sublethal doxorubicin concentrations can be overcome by overexpressing CHK/MATK or inactivating the MMP-2 gene. Consequently, we propose that intracellular/nuclear MMP-2 represents an additional target as it affects the gene and protein expression of the tumor suppressor CHK/MATK in osteosarcoma. We hypothesize that inhibiting intracellular/nuclear MMP-2, resulting in CHK/MATK overexpression, will improve clinical outcomes in osteosarcoma patients treated with lower doses of doxorubicin.

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Conflicts of interest

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Figure legends

Figure 1. Effect of knocking out MMP-2 gene on osteosarcoma cell migration . a. Left; fold change MMP-2 gene expression for U2OS WT and MMP-2 KO cells. Right; MMP-2 protein and activity levels for WT and MMP-2 KO cells, including excreted MMP-2 in cultured OPTI-MEM medium, validating our MMP-2 KO cells. b. Above; transwell migration assay of U2OS WT, MMP-2 KO, MMP-2 KO cells with exogenous MMP-2, and MMP-2 KO cells with WT cultured media containing secreted MMP-2. Below; quantification of number of cells migrated after 24 hours. c. Left; wound closure assays of U2OS WT and MMP-2 KO (n=3). Right; quantification of % wound closure at 24 and 48-hour time-points. Inactivating the MMP-2 gene significantly impeded the migration of U2OS cells, and the addition of exogenous or secreted active MMP-2 did not recover their migratory phenotype.

Figure 2. Role of MMP-2 in enhancement of cell migration by sublethal concentrations of doxorubicin. a. Left; percent cytotoxicity of doxorubicin at various concentrations (0, 0.2, 0.4, 0.6, and 1 μM). Right; percent cytotoxicity of doxorubicin at 48 hours, showing 0.4 μM doxorubicin as a sublethal concentration of doxorubicin for U2OS cells. b. Left; wound closure assays for U2OS WT -/+ 0.4 μM doxorubicin at 24 and 48-hours. Right; quantification of % wound closure at 24 (upper) and 48 (lower) hour time-points (n=3). c. Left; wound closure assays for U2OS MMP-2 KO -/+ 0.4 μM doxorubicin at 24 and 48 hours. Right; quantification of % wound closure at 24 (upper) and 48 (lower) hour time-points (n=3). Although the sublethal concentration of 0.4 μM doxorubicin enhances U2OS cell migration, this enhancement in cell migration is lost when MMP-2 gene is inactivated.

Figure 3. MMP-2 regulates Src phosphorylation at Tyr-416. a. Top; levels of Src phosphorylation at Tyr-416 and Tyr-527 after 0 and 0.4 μM treatment at 24 hours for U2OS WT. Bottom; quantification of pSrc at Tyr-416 (left) and pSrc at Tyr-527 (right) for U2OS WT.

b. Top; levels of Src phosphorylation at Tyr-416 and Tyr-527 after 0 and 0.4 μM treatment at 24 hours for U2OS MMP-2 KO. Bottom; quantification of pSrc at Tyr-416 (left) and pSrc at Tyr-527 (right) for U2OS

MMP-2 KO (n=3). * p<0.05 in comparison to untreated WT control.

In the presence of a sublethal concentration of doxorubicin, Src activation and phosphorylation at Tyr-416 occurs in an MMP-2-dependent manner.

Figure 4. MMP-2 knockout upregulates expression of Src Family Kinase inhibitors. a. Fold gene expression of endogenous Src kinase inhibitors (CHK/MATK, Csk, CDC2), showing significant upregulation of CHK/MATK in U2OS MMP-2 KO, compared to WT cells (*p<0.05). b. Top; protein levels of CSK and CHK/MATK in U2OS WT and MMP-2 KO cells. Bottom; quantification of CSK and CHK/MATK protein levels to ?-actin levels in WT and MMP-2 KO cells (n=3). c. Top; protein levels of CSK and CHK/MATK in 0.4 μM doxorubicin treated U2OS WT and MMP-2 KO cells. Bottom; quantification of CSK and CHK/MATK protein levels to ?-actin levels in 0.4μM doxorubicin treated U2OS WT and MMP-2 KO cells. *p<0.05. Loss of MMP-2 gene caused significant re-expression of the endogenous SFK inhibitor, CHK/MATK.

Figure 5. Overexpression of CHK/MATK impedes osteosarcoma cell migration in the presence of a sublethal concentration of doxorubicin.a. Left; GFP-expression of U2OS WT + GFP-MATK stables, in comparison to U2OS WT, in both GFP and bright field (Scale bar = 200 μm). Middle; gene fold expression for CHK/MATK in WT+GFP-MATK compared to WT. Right; protein expression for CHK/MATK in WT and WT+GFP-MATK, validating expression in WT+MATK stables. b. Top; wound closure assays comparing untreated U2OS WT and U2OS WT+MATK stables, showing complete closure at 48 hours. Bottom; quantification of % of wound closure of WT and WT + MATK stables at 24 and 48 hours (n=3). c. Top; wound closure assays for 0.4 μM doxorubicin treated U2OS WT and U2OS WT+MATK stables. Bottom; quantification of % wound closure of U2OS WT and U2OS WT+MATK stables at 24 and 48-hour time points, showing significant inhibition of cell migration in the WT+MATK stables when treated with 0.4 μM doxorubicin.

Figure 6. Graphical abstract. Left; MMP-2 gene represses CHK/MATK expression. This suppression allows for activation of Src by sublethal concentrations of doxorubicin. Right; inactivation of MMP-2 gene allows for re-expression of CHK/MATK by its turn inhibit doxorubicin-induced Src activation and cell migration.

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