Chondrosarcoma models : understanding chemoresistance mechanisms for use in targeted treatment

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Chapter 7

Src Kinases in Chondrosarcoma Chemoresistance and Migration: Dasatinib Sensitizes to Doxorubicin in TP53 Mutant Cells

This chapter is based on the manuscript: van Oosterwijk JG, van Ruler MAJH, Briaire- de Bruijn IH, Herpers B, Gelderblom H, van de Water B, Bovée JVMG *Br J Cancer. 2013;109(5):1214-22*

Abstract

Chondrosarcomas are malignant cartilage-forming tumors of bone. Due to their resistance to conventional chemotherapy and radiotherapy currently no treatment strategies exist for unresectable and metastatic chondrosarcoma. Previously, PI3K/AKT/GSK3β and Src kinase pathways were shown to be activated in chondrosarcoma cell lines. Our aim was to investigate the role of these kinases in chemoresistance and migration in chondrosarcoma in relation to TP53 mutation status.
We used 5 conventional and 3 dedifferentiated chondrosarcoma cell lines and investigated the effect of PI3K/AKT/GSK3β pathway inhibition (enzastaurin) and Src pathway inhibition (dasatinib) in chemoresistance using WST assay and Live cell imaging with AnnexinV staining. Immunohistochemistry on tissue microarrays (TMAs) containing 157 cartilaginous tumors was performed for Src family members. Migration assays were performed with the RTCA xCelligence System.
Src inhibition was found to overcome chemoresistance, to induce apoptosisand to inhibit migration. Cell lines with TP53 mutations responded better to combination therapy than wildtype cell lines (*p=0.002*). TMA immunohistochemistry confirmed active Src (pSrc) signaling, with Fyn being most abundantly expressed (76.1%).
These results strongly indicate Src family kinases, in particular Fyn, as a potential target for the treatment of inoperable and metastatic chondrosarcomas, and to sensitize for doxorubicin especially in the presence of TP53 mutations.

Introduction

Chondrosarcoma is a malignant cartilage-forming neoplasm of bone and the second most common bone sarcoma in humans (1). Conventional chondrosarcoma does not respond to existing chemo- and radiotherapy modalities (2). Metastasis formation eventually occurs in 71% of grade III chondrosarcoma cases, andwith a 10 year survival rate of 29% this poses a serious treatment problem (3).
Chemoresistance in chondrosarcoma has long been ascribed to poor vascularization, hyaline extracellular matrix production and slowly dividing cells (4;5). Though this is true for low grade chondrosarcomas, high grade chondrosarcomas typically are composed of rapidly dividing cells with more myxoid matrix production (2;6). In the search for molecular targets, negative regulators of the apoptotic pathway, such as BCL-2 (7-10), and survivin (11), were identified to be upregulated in chondrosarcoma, and shown to play a role in chemoresistance (11;12).
Apart from defective apoptotic pathways, deregulated kinase pathways are of growing interest in the field of cancer and have been suggested to play a role in chondrosarcoma (6). We have previously shown activating hyperphosphorylation

of AKT, and Src family kinases and inactivating hyperphosphorylation of GSK3β using kinome profiling of chondrosarcoma cell lines and primary cultures (13).
Both PI3K/AKT/GSK3β and Src signaling pathways are described in a variety of different cancer types as well as in progression to malignancy (14-17) and can be activated by receptor tyrosine kinases (RTKs) (18-20). Activation of the Src pathway promotes cell survival, proliferation, and migration, but can also activate the PI3K/AKT/GSK3β pathway through phosphorylation of PI3K, thereby leading to increased AKT phosphorylation (21). Activation of Protein Kinase C (PKC) by RTKs can also activate the PI3K/AKT pathway, either through phosphorylation of PI3K or through direct phosphorylation of AKT (22;23) (figure 7.1A). Moreover, PKC and AKT can both phosphorylate GSK3β at Ser9 (19;24).
Due to the intricate interplay of PI3K/AKT/GSK3β and Src signaling pathways in cancer and the observation that both pathways are activated in chondrosarcoma we hypothesized that the activation of these pathways in chondrosarcoma contributes to chemoresistance
We therefore investigated the role of both pathways in cell proliferation and chemoresistance. Our data indicate that Src family kinases, Fyn in particular, play a role in chemoresistance and cell migration, and that TP53 mutated cells are especially sensitive to combination therapy with doxorubicin and the Src inhibitor dasatinib.

Methods

Compounds
Doxorubicin and cisplatin were obtained from the in-house hospital pharmacy in a 0.9% NaCl solution. Therapeutic concentrations of doxorubicin in patients are 5-50μM with an *in vitro* range of 1-10 μM, for cisplatin these are 3-13μM with an *in vitro* range of 1-50μM (25). The PKC inhibitor enzastaurin (26) (Eli Lilly, IN, USA) and the Src inhibitordasatinib (27) (Bristol-Meyers Squibb, Princeton, NJ, USA) were dissolved in DMSO.

Cell culture
Chondrosarcoma cell lines (table 7.1), as well as MCF-7 and HeLa cell lines were cultured in RPMI1640 (Gibco, Invitrogen Life-Technologies, Scotland, UK) supplemented with 1% L-glutamax, 1% penicillin/streptomycin (100U/mL), and 10% heat-inactivated Fetal Calf Serum (Gibco, Invitrogen Life-Technologies, Scotland, UK). Cells were grown at 37°C in a humidified incubator with 95% air and 5% CO_2. Cells were cultured until stably multiplying.Chondrogenic phenotype was confirmed using RT-PCR for collagen I, IIB, III, and X, aggrecan, and SOX9(28). Identity of cell lines was confirmed using the Cell ID^{TM} System after completion of experiments (Promega Benelux BV, Leiden, The Netherlands).

Table 7.3. Cell lines

Cell Line	Tumor Type	Grade	Gender	Age	Passage	TP53[1]	IDH1[2]	IDH2[2]	Reference
SW1353	Solitary Central	II	F	72	21	V203L	wt	R172S	ATCC
OUMS27	Solitary Central	III	M	65	27	wt	wt	wt	(58)
CH2879	Solitary Central	III	F	35	>80	wt	wt	wt	(59)
JJ012	Solitary Central	II	M	39	9	G199V	R132G	wt	(60)
L835	Solitary Central	III	M	55	50	wt	R132C	wt	(12)
L2975	Dedifferentiated CS		M	57	60	wt	R172W	wt	(12)
NDCS1	Dedifferentiated CS		M	38	60	C242S	wt	wt	(61)
L3252	Dedifferentiated CS		F	52	30	wt	wt	wt	(12)

[1]IDH mutations for used cell lines were described in (12;30)
[2]TP53 mutations for used cell lines were described in (12;29)

Cell viability assay
Chondrosarcoma cell lines were plated in 96 well plates for viability assessment ($2x10^4$-$2x10^5$ cells/well depending on growth rate) and allowed to grow and adhere overnight after which the respective drugs were added in their corresponding concentrations. Combination assays were performed as described (29) with alternating treatments combining enzastaurin, dasatinib, and/ or doxorubicin. All experiments were performed in triplicate and at least three times. Graphs show data from one representative experiment. Error bars indicate the standard deviation.

Immunoblotting
Immunoblotting using AKT, pAKT, Fyn (Cell Signaling, Leiden, the Netherlands) and pSrc antibody (pSrc pY418, Invitrogen Life Technologies, Bleiswijk, the Netherlands) to investigate the Src and PI3K/AKT signaling pathway and p53 (Do7, Dako, Heverlee, Belgium), MDM2 (IF2, Zymed, Bleiswijk, the Netherlands) and p21 (Santa Cruz, Heidelberg, Germany) was performed as previously described (13), using 20μg of each sample.

Mutation analysis
To identify mutations in AKT1, direct sequencing was performed as described (30), using DNA derived from 57 tumors, 8 cell lines, and 1 primary culture (L3310) using forward primer 3'-TAGAGTGTGCGTGGCCTCTCA-5' and reverse primer 3'-CTGAATCCCGAGAGGCCAA-5' to screen for hotspot mutations in the AKT1-E17K pleckstrin homology domain.

Apoptosis assay and Immunofluorescence
Apoptosis assay and immunofluorescence for caspase 3 and cytochrome C were performed as described (29;31). In short, 20.000 chondrosarcoma cells were grown in black 96-well microclear plates (Greiner®, Sigma-Aldrich, Zwijndrecht, The Netherlands) to perform a live cell apoptosis assay (31), with AnnexinV-Alexa633 conjugate using the BD Pathway® 855 (Becton Dickinson, Breda, The Netherlands). Time series were quantified using in house developed macros for Image-Pro Plus (Media Cybernetics, Bethesda, USA). Drugs were added 0, 24, and 48 hours before imaging and Annexin V-Alexa633 conjugate was added immediately prior to imaging. For all treatments, a pan-caspase inhibitor, z-VAD-fmk (Bachem-Holding AG, Weil am Rhein, Germany), was added 30 minutes before drug addition and imaging in order to establish apoptosis specificity of the assay. Prior to imaging, live nuclei were stained with HOECHST-33342 at 100ng/ml. All experiments were performed in triplicate and at least three times. Error bars show standard deviation from one representative experiment.

Migration assays
The RTCA xCelligence system (Roche Applied Sciences, Almere, the Netherlands), based on cell-electrode substract impedance detection technology, was used for migration assays. For migration assays, lower wells of the SIM plates (migration plates) were filled with growth medium (20% FCS in RPMI).Cell lines were plated at a density of 80.000 cells per well in the top wells in empty buffer (RPMI only) containing0, 0.2, 0.4, 0.6, 0.8, or 1.0 μM dasatinib.SIM plates were loaded into the RTCA station in the cell culture incubator immediately after plating and cell index was acquired every 5 minutes. Cell index as acquired by the software was set to 100% migration after flattening of the slope. Experiments were performed in triplicate.

TMA construction and clinicopathological data
Tissue microarrays (TMAs) were constructed from formalin-fixed, paraffin-embedded tissue using standard procedures (32) using a 2.0 mm diameter punch automated tissue arrayer (3DHistech Ltd, Budapest, Hungary). Each array contained three cores per tumor wherever possible including 7 control tissues (skin, colon, tonsil, prostate, mamma carcinoma, spleen and liver). Using a tape-transfer system (Instrumedics, Hackensack, NJ, USA), 4-μm sections were transferred to glass slides.All specimens in this study were handled according to the ethical guidelines described in "Code for Proper Secondary Use of Human Tissue in The Netherlands" of the Dutch Federation of Medical Scientific Societies. 157 patients with cartilaginous tumors were selected from the archives of the Leiden University Medical Centre. Selected cases included 137 conventional chondrosarcomas (central chondrosarcoma, n=92; peripheral chondrosarcoma, n=45) and 20 benign cartilage tumors (osteochondroma, n=9; enchondroma, n=11). Only primary tumors were selected. Histology was reviewed by an experienced bone tumor pathologist (J.V.M.G.B.). Clinicopathological data are shown in table 7.2.Total follow up was available for 136 of 157 patients, with 14 patients showing metastasis at completion of this study. Histological grading of chondrosarcoma was performed according to Evans (3). Rare chondrosarcoma subtypes were excluded.

Table 7.4 Clinicopathological data

	Peripheral (n =45)	Central (n =92)
Male vs female	27 vs 18	39 vs. 53
Median age at diagnosis	37 (14-82)	50 (20-84)
Histology		
Grade I	31	42
Grade II	11	36
Grade III	3	14
Metastasis	4/45	10/92
Median follow-up (months)	121 (15-299)	103 (7-292)

Immunohistochemistry

Immunohistochemistry was performed on the TMAs. Slides were incubated with antibodies against Src, Lck, Fyn, Yes, and phosphorylated Src (pSrc, recognizes active Src family members phosphorylated at Y419). Details of antibodies and procedures are provided in table 7.3. Immunohistochemical reactions were performed according to standard laboratory methods (7) and visualized using DAB+ Substrate Chromogen System (Dako, Heverlee, Belgium). TMA slides were scanned using a high resolution Mirax Desk Intrument (Zeiss, Mirax 3D Histech, Hungary) and scored independently by two observers (JVMGB and JGvO) and discrepancies were discussed. Staining intensity (0 = absent, 1 = weak, 2 = moderate, 3 = strong) and extent of the staining (0 = 0%, 1 = 1-24%, 2 = 25-49%, 3 = 50-74% and 4 = 75%-100%) were assessed. Staining was considered high (score ≥4) or low (score <4). As external positive and negative control for all the antibodies specimens of normal tonsil were used. Cores with a negative internal control or loss of tissue were excluded from the analysis.

Statistical analysis
Survival was evaluated by Kaplan–Meier analysis and the log-rank test. Values of $p \leq 0.05$ were considered statistically significant. Variables that achieved significance ($p \leq 0.05$) were entered subsequently into a multivariate analysis using the Cox regression model. Cox regression analysis was carried out with clinical outcome (overall survival) as the independent variable. Correlation between expression and grade and individual stainings were evaluated using Pearson chi-squared test for independent variables. Values of $p \leq 0.05$ for asymptomatic 2 sided testing were considered significant. The data were analyzed using SPSS version 17.0 software (Chicago, IL, USA).
For combination assays the combination index according to the method of Chou and Talalay (33) was calculated. A combination index (CI) of below 1 indicates synergy, and CI of above 1 indicates additive effect. Correlation between combination indices was evaluated using independent 2 sided t-test using Graphpad Prism 5 software (La Jolla, CA, USA). Values of $p \leq 0.05$ were considered significant.

Table 7.3. Antibodies used for immunohistochemistry

Antibody	Clone	Dilution	Antigen Retrieval	Blocking	Source
Src	*327554*	*1:200*	*Citrate*	*NGS*	*R&D Systems Europe Ltd, Oxon, UK*
Yes	*339827*	*1:4000*	*Citrate*	*Milk*	*R&D Systems Europe Ltd, Oxon, UK*
Lck	*Y123*	*1:250*	*Citrate*	-	*Abcam, Cambridge, UK*
Fyn	*Y303*	*1:30000*	*Citrate*	*Milk*	*Abcam, Cambridge, UK*
pSrc	*AF2685*	*1:200*	*Citrate*	*Milk*	*R&D Systems Europe Ltd, Oxon, UK*

Results

PI3K/AKT/GSK3β pathway is not involved in chemoresistance of chondrosarcoma cell lines
To investigate the PI3K/AKT/GSK3β pathway, chondrosarcoma cells were treated with 1μM and 10μM enzastaurin (26), a PKCβ inhibitor shown to inhibit AKT signaling and GSK3β phosphorylation (34). Whereas the cervical cancer cell line HeLa shows 70% reduction in cell viability after treatment with 10μM enzastaurin (fig 7.1B), chondrosarcoma cell lines were less sensitive to enzastaurin treatment. Two chondrosarcoma cell lines showed complete resistance (NDCS-1 and L2975) while in the two most responsive cell lines (SW1353 and L3252) a maximum reduction in cell viability of ~40% was achieved (fig 7.1B). As the PI3K/AKT/GSK3β pathway is involved in cell survival, we set out to examine its role in chemoresistance. Enzastaurin was combined with doxorubicin over the course of 72hrs, alternating treatments every 24hrs, as we previously showed that drug

administration on separate days was most effective (29). While there was no difference in response between IDH mutated and IDH wildtype cell lines, cell lines with TP53 mutations responded better to combination treatment than TP53 wildtype cell lines (*p=0.002*) (fig 7.1B) . However, a lack of synergy between the two drugs was observed (combination indices >2), as reduction in cell viability was attributed to the effect of doxorubicin alone (NDCS-1) or the additive effect of enzastaurin and doxorubicin. Activation of AKT1 can be through mutations in the pleckstrin homology domain, found mostly in solid tumors (35), leading to activated downstream signaling and decreased sensitivity to kinase inhibitors (36). Hotspot mutations in the pleckstrin homology domain of AKT1 were absent in the primary chondrosarcoma tumor tissues or cell lines.

Inhibition of Src family kinases with dasatinib does not potentiate the effect of enzastaurin in chondrosarcoma cell lines

To exclude active Src signaling causing the limited response we observed to enzastaurin we combined enzastaurin with the Src inhibitor dasatinib. In five cell lines (CH2879, OUMS27, SW1353, NDCS-1, and L3252) cell viability after combination treatment dropped below 50% (fig 7.1C). However, the reduction in cell viability could not be ascribed to a synergistic effect in any of the cell lines. Rather it was found to be due to the effect of dasatinib (L835, NDCS-1 and L3252) or the additive effect of dasatinib and enzastaurin (combination indices >2, fig 7.1C). TP53 mutation status was not correlated to response (*p=0.38*, fig 7.1C). Interestingly, treatment with 1μM dasatinib for 24hrs was found to decrease phosphorylation of AKT in OUMS27, L835, L3252 and NDCS-1 cell lines (fig 7.2A).

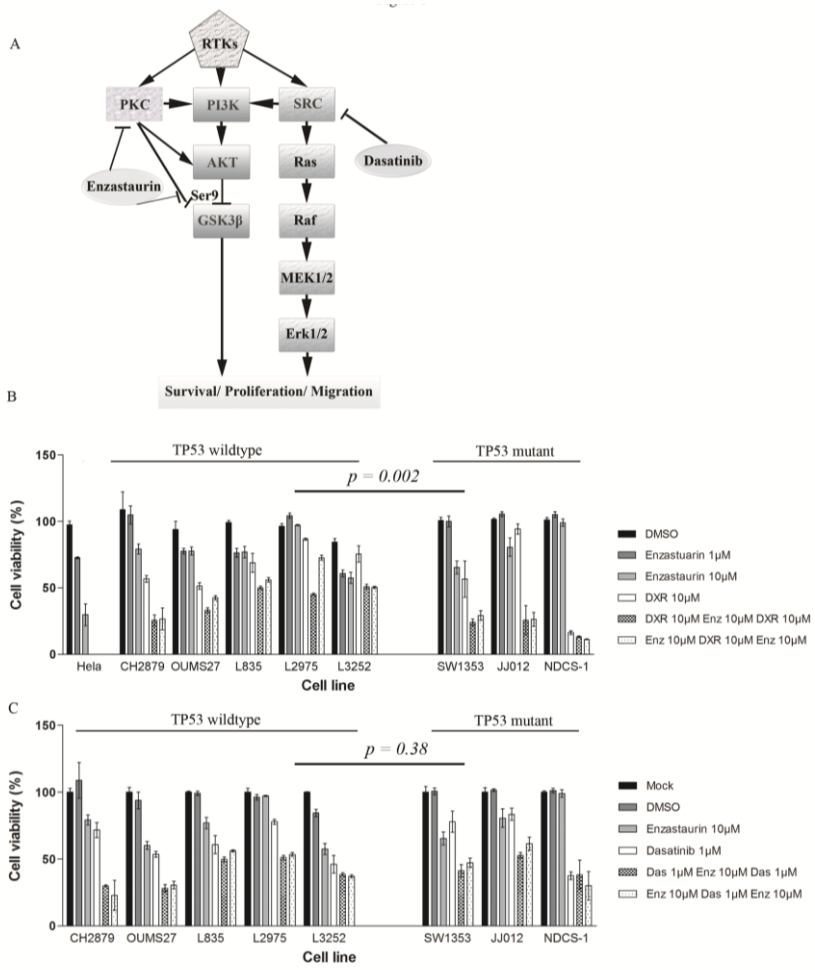

Figure 7.1. Chondrosarcoma cell lines are not sensitive to PKC inhibition. A: Schematic representation of activation of PI3K and Src pathway by receptor tyrosine kinases (RTKs). RTKs can activate protein kinase C (PKC), phosphatidylinositol 3-kinase (PI3K), and Src. PKC and Src can also activate the PI3K/AKT/GSK3β pathway, promoting survival, proliferation, and migration. The Src pathway activates the Ras/Raf pathway. Enzastaurin is a selective PKC inhibitor also reported to inhibitinactivating phosphorylation of GSK3β. Dasatinib is a Src inhibitor. Adapted from Fizazi (62).B: HeLa cell line showing 70% decrease in cell viability after treatment with enzastaurin. Chondrosarcoma cell lines poorly respond to enzastaurin alone, and an additive effect is observed when alternating 10μM doxorubicin (DXR) and 10μM enzastaurin (Enz) for 24hrs each for 72hrs in total. No difference is observed when order of administration is reversed. Significant difference between TP53 mutant and wildtype cell lines (*p=0.002*).C: Combination of enzastaurin with Src inhibitor dasatinib (Das) showing additive effect in chondrosarcoma cell lines. No significant difference is observed between TP53 mutant and wildtype cell lines (*p=0.38*).

Src signaling contributes to chemoresistance of chondrosarcoma cells
We have previously shown Src signaling to be involved in chondrosarcoma cell proliferation (13). Immunoblotting confirmed the presence of phosphorylated Src (Y418) in the chondrosarcoma cell lines, with lowest expression in L835 cells, and 24hrs with 1µM dasatinib resulted in decreased pSrc levels (Fig 7.2A). To examine the role of Src signaling in chemoresistance, dasatinib was combined with doxorubicin. A synergistic effect was observed in cell lines CH2879, OUMS27, SW1353, JJ012, NDCS-1, and L2975 (combination indices ranging from 0.09 to 0.88 fig 7.2C), and the order of drug administration did not influence efficacy. Interestingly, a significant difference between both the cell viability (*p=0.002*) and the combination indices (*p=0.043*) was observed between cell lines with and without TP53 mutations, and both cell lines that were resistant to combination treatment (L835 and L3252) were wildtype for TP53 mutations. We continued to investigate p53 accumulation as well as MDM2 and p21 expression in cells treated with and without treatment with dasatinib (fig 7.2B). As expected, high p53 protein expression with low to absent p21was seen in the three TP53 mutant cell lines. All TP53 wildtype cell lines demonstrated low p53 and p21 protein expression with the exception of CH2879, demonstrating high levels of p53 and p21. Protein levels were not affected by dasatinib treatment. All cell lines showed low MDM2 protein expression. No correlation with IDH mutations was found.

Src inhibition combined with doxorubicin induces apoptosis
Using annexinV binding live cell imaging we confirmed our previous findings (13) that dasatinib monotreatment does not induce apoptosis (fig 7.2D first 24 hours). However, when combined with doxorubicin, up to 50% of cells had entered apoptosis after completion of the third cycle of combination treatment; (JJ012 cell line shown as representative cell line, fig 7.2D). Due to the effect of doxorubicin during the first 24 hours, 10% more cells had entered apoptosis during combination treatment starting doxorubicin, than during combination treatment starting with dasatinib. Apoptosis could be inhibited using the pan-caspase inhibitor zVADfmk (results not shown).

Dasatinib inhibits migration of chondrosarcoma cell lines
As Src family members also play a role in motility and adhesion (37), we continued to investigate the migratory capacity of the chondrosarcoma cell lines. Using a transwell system, all chondrosarcoma cell lines showed migratory properties, and started migrating approximately 30 minutes after plating, except for JJ012 cells, which started migrating only 4 hours after plating (results not shown). In the presence of dasatinib, however, a complete inhibition of cell migration was achieved for all cell lines at concentrations as low as 200nM (fig 7.2E, F). No difference between cell lines harboring TP53 mutations and wildtype cell lines was observed.

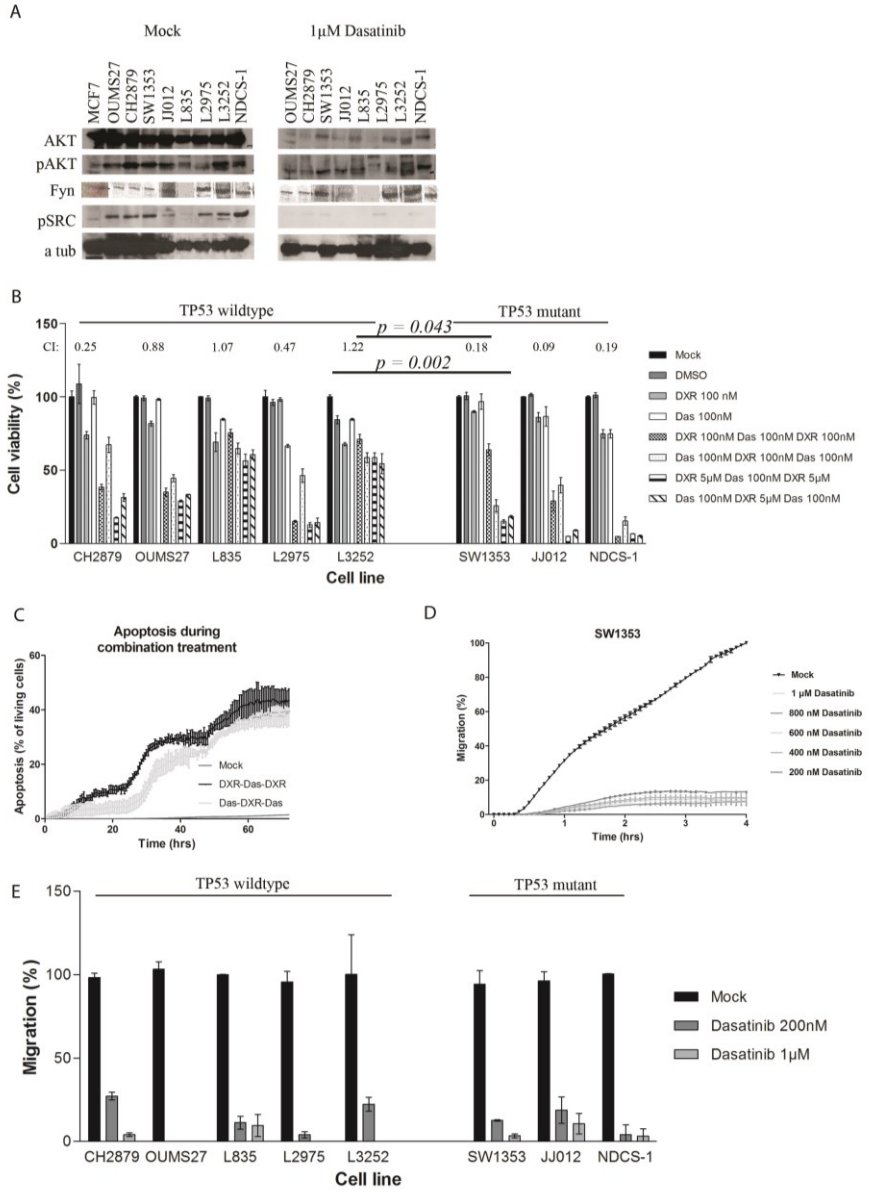

Figure 7.2. The Src pathway is involved in chondrosarcoma chemoresistance.
A: Immunoblotting showing AKT, pAKT, Fyn, pSrc and loading control α-tubulin (a tub) for untreated chondrosarcoma cell lines and after 24 hrs 1µM dasatinib (Das). MCF-7: breast cancer cell line, shown as positive control. Presence of all kinases in all cell lines. phophorylated Src in all cell lines, although levels are very low in L835. After 24hrs 1µM

dasatinib treatment, levels of pSrc (at Y418) are decreased in all cell lines, and of pAKT in OUMS27, L835, L3252, JJ012, and NDCS-1. B: Immunoblotting showing p53, MDM2, and p21 in untreated chondrosarcoma cell lines (Mock) and after 24hrs 1µM dasatinib (Das). U2OS (osteosarcoma) cell line is shown as positive control. MDM2 expression is low in all cell lines. TP53 wildtype cell lines are negative for p53 protein expression, with low p21 protein expression, except for CH2879. TP53 mutant cell lines show high TP53 protein expression with low p21 protein expression. No change in protein levels is observed after 24hrs 1µM dasatinib treatment. C: Combination of dasatinib (Das) with doxorubicin (DXR) leads to synergistic loss of cell viability at concentrations which are ineffective on their own in most cell lines. Combination treatment was more effective in TP53 mutant cell lines than in TP53 wildtype cell lines (*p=0.002* for cell viability, *p=0.043* for combination indices). D: Apoptosis assay in JJ012 cell line alternating 1µM dasatinib (Das)and 1µM doxorubicin (DXR)demonstrates the occurrence of apoptosis during combination. Apoptosis is calculated as percentage of AnnexinV-Alexxa633 stained cells per total number of HOECHST stained cells. D, E: Dasatinib successfully inhibits migration in chondrosarcoma cell lines in concentrations as low as 200nM. E: SW1353 cell line shown as representative over the course of 4 hrs, F: bar chart showing migration for all cell lines.

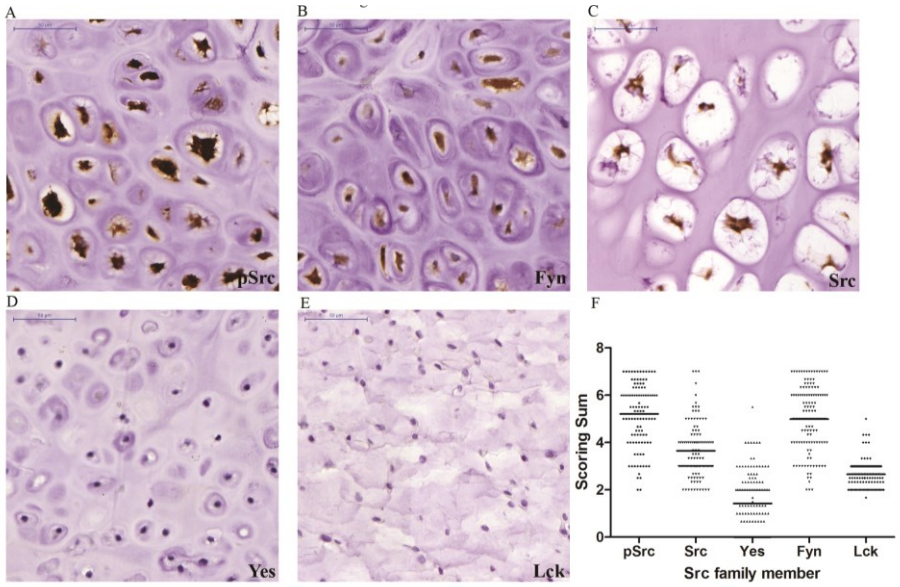

Figure 7.3. Immunohistochemistry demonstrating expression of Src family members in conventional chondrosarcoma tissue.
A: High pSrc expression in grade I chondrosarcoma. B: High intensity nuclear FYN expression in grade I chondrosarcoma. C: High intensity cytoplasmic and nuclear Srcexpression in grade I chondrosarcoma. D: Absence of Yes expression in grade II chondrosarcoma. E: Absence of Lck in grade II chondrosarcoma. Scale bars: 50µm. F: Scatterplot showing distribution of staining scores among chondrosarcoma tissue samples.

Fyn is the most important Src family member in chondrosarcoma tissues
To identify the most important Src family member in chondrosarcoma, we evaluated the expression of the 4 family members Src, Yes, Fyn and Lck, as well as pSrc in primary tumor samples. Active Src signaling as evidenced by positive staining for pSrc was found in 88-100% of the tumors (table 7.4, fig 7.3A, F).Of the 4 Src family members we found Fyn (76.1% high expression (89/117)) and Src (46.6% high expression (48/103)) to be most abundantly expressed in chondrosarcoma (table 7.4, figure 7.3B, C, F). In contrast, high expression of Yes and Lck was observed in only 5% of all chondrosarcoma cases (6/116 and 6/120 respectively) (table 7.4, figure 7.3D, E, F). During the staining procedures some cores were lost due to inherent structural instability of the tissue. A significant increase in Src expression was seen between grade I and grade II peripheral CS (p=0.005 Pearson chi squared test). Though not significant, Src expression in tumors was found to be inversely correlated with overall survival (p=0.3 log rank). Fyn expression was found to significantly increase with increasing histological grade in both peripheral chondrosarcoma (p= 0.05 Pearson chi squared test) and central chondrosarcoma (p= 0.000 Pearson chi squared test). No significant correlations to metastasis were found. Using western blot we confirmed expression of FYN in all cell lines, with low expression in L835 (fig 7.2A)

Discussion

Chondrosarcomas are resistant to conventional chemotherapy. Despite ongoing research, there is still nothing to offer patients with unresectable or metastatic disease and the need for new, targeted therapies is high. We here explored the effects of increased PI3K/AKT/GSK3β and Src signaling on chondrosarcoma chemoresistance and cell migration using enzastaurin and dasatinib, respectively. We show that dasatinib is more effective in overcoming chondrosarcoma chemoresistance than enzastaurin, and acts synergistically with doxorubicin to inhibit cell viability and induce apoptosis. Most importantly, we show that in cell lines with TP53 mutations, the combination of tyrosine kinase inhibitors with doxorubicin is more beneficial than in wildtype TP53 cell lines.
Chondrosarcoma is a heterogeneous disease, and this heterogeneity is represented in the cell lines. Recently, IDH1 and IDH2 mutations were found in chondrosarcoma (38), and we published that these mutations are retained in chondrosarcoma cell lines (12;30). Of the two cell lines that were nonresponsive to combination treatment of doxorubicin with dasatinib, one central chondrosarcoma cell line (L835) carried an IDH1 mutation, whereas the other (dedifferentiated chondrosarcoma cell line (L3252)) was wildtype for IDH. Thus, no correlation between IDH mutation status and response to dasatinib monotreatment or combination treatment with doxorubicin was observed. More likely, the lack of

sensitivity to dasatinib in the L835 cell line is caused by the low pSrc activity in this cell line.

Src inhibition with dasatinib resulted in successful sensitization for doxorubicin treatment, especially in TP53 mutant chondrosarcoma cell lines. Approximately 30% of chondrosarcomas carry TP53 mutations, and these mutations are found especially in high grade chondrosarcomas (39;40). Three of the eight cell lines carry a TP53 mutation (SW1353, JJ012, and NDCS-1), and these cell lines also showed a better response to combination treatment with low combination indices when compared to TP53 wildtype cell lines. This is an interesting result as mutant TP53 is described to actively inhibit apoptosis through activation of p21 (41) or to confer chemoresistance through engaging in oncogenic transcription complexes (42). Previously, dasatinib was found to interfere with the p53 transcriptional activity induced by the MDM2 inhibitor nutlin-3(43). We show that dasatinib does not affect p53 nor p21 protein expression in chondrosarcoma cells. Dasatinib as a single agent proved ineffective in chondrosarcoma patients (Schuetze, CTOS 2010). However, recent clinical studies with dasatinib in other malignancies have shown its efficacy not only irrespective of TP53 status as a single agent (44), but also to overcome TP53 mutation status related chemoresistance (45). The results of these clinical studies in combination with the data we show here strongly suggest clinical evaluation of the efficacy of dasatinib in combination with doxorubicin in chondrosarcoma patients harboring TP53 mutations.

Since we demonstrate Src signaling to play a role in chemoresistance, we further explored the expression of the different Src family kinases (SFKs) in human chondrosarcoma tissues. Fyn was most widely expressed (89/117) and was found to increase with increasing histological grade, suggesting a role in chondrosarcoma progression. Fyn is reported to be upregulated in multiple cancers, and to be associated with malignant progression and metastasis formation (37;46;47). We confirmed that indeed the Src pathway is important in chondrosarcoma cell motility, since dasatinib completely inhibited migratory capacity of all chondrosarcoma cell lines even at low dose.

Clinical trials with dasatinib have shown the efficacy and low toxicity of dasatinib in combination with conventional chemotherapeutic agents in solid tumors (48). In a phase II study of dasatinib with hyper-CVAD in patients with Philadelphia chromosome positive lymphoblastic leukemia, long term remission was achieved in newly diagnosed patients (49), and in a phase I-II study of dasatinib with doxetaxel in castration resistant prostate cancer, disappearance of bone lesions was obtained (50). The results obtained with dasatinib in combination with chemotherapy strongly encourage the exploration of dasatinib in combination with doxorubicin in patients with chondrosarcoma.

Table 7.4 Protein expression in tumors using immunohistochemistry

	Osteochondroma	**Peripheral Chondrosarcoma**			**Enchondroma**	**Central Chondrosarcoma**		
		Grade I	**Grade II**	**Grade III**		**Grade I**	**Grade II**	**Grade III**
pSrc	3/3 (100%)	12/13 (92%)	6/6 (**100**%)	3/3 (**100**%)	4/5 (80%)	21/24 (88%)	21/22 (96%)	10/10 (**100**%)
Src	1/7 (14%)	7/17 (41%)	8/8 (**100**%)	2/3 (67%)	4/7 (57%)	12/31 (39%)	12/31 (39%)	7/13 (54%)
Yes	0/7 (0%)	3/23 (13%)	0/10 (0%)	0/3 (0%)	0/7 (0%)	0/36 (0%)	3/30 (10%)	0/14 (0%)
Fyn	7/8 (88%)	16/20 (80%)	9/10 (90%)	3/3 (**100**%)	5/5 (**100%**)	17/35 (49%)	31/35 (89%)	13/14 (93%)
Lck	0/3 (0%)	1/23 (4%)	2/10 (20%)	0/3 (0%)	0/7 (0%)	1/38 (3%)	0/33 (0%)	2/13 (15%)

Both the PI3K/AKT/GSK3β and Src kinase pathways are activated by receptor tyrosine kinases (RTKs) (18;19), and play diverse roles in promoting growth, survival, and metastasis (16-18;20;51). We show here that constitutive activation of AKT due to mutations does not play a role in chondrosarcoma, and further research should elucidate which RTK is responsible for the high AKT, GSK3β, and Src phosphorylation (13). A possible candidate is IGF-1, which can activate the PI3K/AKT and Src pathway through the RTK IGF-1R (52), and has been shown to induce PI3K/AKT signaling and migration in chondrosarcoma cell lines (53). Src family kinases can induce phosphorylation of the RTK domains of IGF-1 as well as the PDGF receptors through SHP-2 leading to receptor internalization. This increases binding efficacy with PI3K, leading to increased proliferative capacity of cancer cells (54;55). Moreover, AKT functions as a gatekeeper of apoptosis through phosphorylation of BAD. AKT mediated phosphorylation of BAD inhibits its binding capacity to antiapoptotic BCL-2 family members, which will prevent a cell from entering apoptosis (56;57). We recently published that the antiapoptotic BCL-2 family members also play a role in chondrosarcoma chemoresistance (29). Combined with the results of the present study, this is suggestive of a common mechanism. However, more studies are needed to explore whether the activation of the IGF pathway by Src leading to the inhibition of BH3 proteins and apoptosis through AKT may be involved in chondrosarcoma chemoresistance.

In conclusion, we found that inhibition of the Src pathway was successful in overcoming chemoresistance and inhibited migration. A synergistic response to combination treatment was observed which was significantly stronger ($p=0.002$) in cell lines harboring TP53 mutations. Moreover, as we observed the Src family member Fyn to be the most prevalent in chondrosarcoma tissues, we hypothesize Fyn to play a major role in the chemoresistance and malignant progression of chondrosarcoma. These results aid in the understanding of signaling pathways in chondrosarcoma and may lead to the development of effective therapeutic strategies for currently untreatable metastatic chondrosarcoma.

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