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miR-138-mediated Regulation of Kindlin-2 Expression Modulates Sensitivity to Chemotherapeutics

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Abstract

Prostate cancer (PCa) is the second leading cause of cancer-related death in men, second only to lung cancer, mainly due to disease reoccurrence as a result to lack of response to androgen deprivation therapies (ADT) after castration. Patients with metastatic castration-resistant prostate cancer (mCRPC) have very limited treatment options, with docetaxel as the first line standard of care, for which resistance to this chemotherapeutic ultimately develops. Therefore, finding ways to sensitize tumors to chemotherapies and to limit chemoresistance provides a viable strategy to extend the survival of mCRPC patients. The present study investigated the role of Kindlin-2 (FERMT2/K2), a member of the Kindlin family of FERM domain proteins and key regulators of the adhesive functions mediated by integrin, in the sensitization of mCRPC to chemotherapeutics. Loss of K2, which is overexpressed in PCa cells derived from mCRPC tumors, compared to those cells derived from androgen-dependent tumors, significantly enhanced apoptosis and cell death of docetaxel-treated PC3 cells. Furthermore, it was determined that K2-mediated sensitization to docetaxel treatment is the result of inhibition of β 1-integrin signaling. Finally, miR-138 specifically targeted K2 and inhibited its expression, thereby regulating a miR-138/K2/ β 1-integrin signaling axis in mCRPC that is critical for the modulation of sensitivity to chemotherapeutics. Thus, these data identify a novel signaling axis where K2 in combination with chemotherapeutics provides a new target for the treatment of mCRPC.

Keywords

Kindlin-2; miR-138; metastatic castration resistant prostate cancer (mCRPC); Chemoresistance; β 1 integrin

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COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

EFP discussed the design of the experiments, the results and assisted in writing. KSA supervised the overall design of the experiments and wrote the manuscript.

INTRODUCTION

Prostate cancer (PCa) is the leading type of cancer in men accounting for more than 25% of all new reported cancer cases (1). Prostate cancer is also the second cause of cancer death in men (1). The 5-year survival rate of patients diagnosed with early stage PCa is more than 98% (1). This high survival rate is credited to the success of early treatments that include surgery, radiation therapy and hormone therapy either alone or in combination. The aim of these treatments is to inhibit androgen:androgen receptor signaling which is the main driver for prostate cancer progression and metastasis (2;3). In some cases, PCa tumors become insensitive to androgen ablation resulting in rapid progression to the metastatic stage, hence the term metastatic castration resistant prostate cancer (mCRPC). Chemotherapy in the form of taxanes such as docetaxel has been used as the first line standard of care for treatment of mCRPC and has been shown to significantly extend the life of patients with mCRPC (4;5). However, for those patients who ultimately develop resistance to docetaxel, there are very limited treatment options, and these patients often succumb to the disease. Combination therapy that is aimed at sensitizing cancer cells to chemotherapeutics to overcome chemoresistance has emerged as an option to treat patients at risk of developing chemoresistance.

In the present study, we found that inhibition of Kindlin-2 (K2) in mCRPC cells significantly enhances their sensitivity to apoptosis and cell death induced by docetaxel. K2 is one of a three member family of FERM domain proteins with a PH subdomain inserted into their F2 subdomain (6). Over the past decade, many lines of evidence have emerged to implicate the Kindlins as important regulators of integrin adhesion receptors (7;8). Specifically, Kindlins appear to cooperate with talin to be essential regulators of the ligand binding functions of integrins and thereby increase the adhesive and migratory responses of cells (6;8), properties that are often enhanced in transformed cells. K2 (FERMT2) is the most broadly distributed of the Kindlins (8). More importantly, in PCa, K2 is highly expressed in the CRPC cell lines compared to androgen-sensitive PCa cells lines (our data and Ref (9)), suggesting a potential role of K2 in the pathology of PCa. Our study reveals that K2 expression levels determine the sensitivity of mCRPC cells to apoptosis and cell death induced by docetaxel; that knockdown of K2 enhances susceptibility of PC3 cells to docetaxel; and that over-expression of K2 enhances resistance of these cells to docetaxel. Mechanistically, we found that K2 knockdown synergizes with docetaxel to significantly enhance the induction of apoptosis and cell death of PCa cells through inhibition of the $\beta 1$ integrin-mediated outside-in signaling manifest by cell spreading. We further show that the ability of K2 to modulate chemosensitivity is regulated by microRNA miR-138, which specifically targets the 3'UTR of K2. Thus, our data identify a novel miR-138 $\leftrightarrow$ K2 $\leftrightarrow$ $\beta 1$ integrin signaling axis that plays a critical role in the modulation of response to chemotherapy and, thereby, suggest K2 inhibitors may be a target for adjunctive therapy to treat drug resistant mCRPC.

EXPERIMENTAL PROCEDURES

Antibodies

Mouse monoclonal anti-K2, clone 3A3(1:2000), was purchased from EMD Millipore (Billerica, Massachusetts); mouse anti-GFP was from Santa Cruz Biotechnology Inc (Santa Cruz, CA); goat horseradish peroxidase-conjugated anti-mouse IgG (1:5000) and goat horseradish peroxidase-conjugated anti-rabbit IgG (1:5000) were from Calbiochem (Billerica, Massachusetts); and Alexa 568-conjugated phalloidin were from Invitrogen. PE-conjugated anti-CD29 and Vecta-shield with 4',6-diamidino-2-phenylindole was from Vector Laboratories (Burlingame, CA). Gel electrophoresis reagents were from Bio-Rad (Hercules, CA).

siRNA and microRNA gene expression knockdown

For transient knockdown of K2 expression, we used the FERMT2 Silencer Select siRNA (Ambion; Austin, Texas) to target the ORF of FERMT2 (siRNA ID #s21614) and Mig-2 siRNA sc-106786C (Santa Cruz) to target the 3'UTR of FERMT2, as previously described (10;11). microRNA miR-138 mimic and miR-138 inhibitor were from Qiagen (SABiosciences; Valencia, CA).

Cell Culture

Human PC3, DU145 and LNCaP PCa cells were purchased from American Type Culture Collection (ATCC). PC3 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) F12 supplemented with 10% fetal bovine serum (FBS), 100 units of penicillin/ml, 100 µg of streptomycin/ml. DU145 and LNCaP cells were maintained in RPMI supplemented with 10% FBS, 100 units of penicillin/ml and 100 µg of streptomycin/ml. For docetaxel treatment, Docetaxel (5 µM) or vehicle (DMSO) was added to culture media and cell were incubated for 12 hrs before further processing.

Real-time quantitative-RT-PCR

Total RNA was extracted from cancer cell lines using TRIzol reagent, following to the manufacturer's (Invitrogen; Grand Island, NY) instructions. cDNA was generated and used as a template for quantitative RT-PCR performed as previously described (11;12). The expression levels of each transcript were normalized using the 2^{-C_t} method (13;14) relative to GAPDH.

Flow cytometry

PC3 cells, transfected with either the control non-targeting siRNA or the siKindlin-2, were propagated in DMEM F12 supplemented with 10% FBS. Subconfluent cell cultures were treated with either the diluent or docetaxel (5 nM) for 12 h, after which cells were detached by trypsinization and washed with Hank's Balanced Salt Solution with Ca²⁺, Mg²⁺, and 0.1% BSA. Cells were processed for flow cytometry as previously described (15;16) and following the manufacturer (Roche In-Situ Cell Death Detection Kit, Fluorescein). Annexin V and propidium iodide was used to quantify apoptotic and dead cells, respectively (16). For the quantification of β1 and β3 integrin surface expression, we used PE mouse anti-human

CD29 and FITC mouse anti-human CD61, and their respective PE and FITC mouse IgG controls (BD Pharmingen; Franklin Lakes), NJ. All data were acquired and analyzed in a BD FACSalibur instrument.

Immunoblot Analysis

We followed the standard procedures for Western blotting as previously described (10–12;15;16). Signals were quantified using the ImageJ software according to the parameters described in ImageJ user guide (<http://rsbweb.nih.gov/ij/docs/guide/146.html>). Average values from 3 different blots are presented.

Cell spreading and immunofluorescence microscopy

These assays were performed as described in our previously published studies (10). Briefly, cell spreading assays were carried out on round coverslips (Fisher Scientific) in 12-well plates (Falcon, Becton Dickinson & Co. Franklin Lakes, NJ). Coverslips were coated for 1 hour with 20 µg/mL of fibronectin diluted in 1X PBS I (Sigma) at 37°C. Following 3 washes with PBS, coverslips were blocked for 1 hour with 1% BSA in PBS at 37°C. BSA (1 mg/ml) coated coverslips were used as negative controls. PC3 cells, 4×10^5 /ml in serum-free media, were treated as specified and then seeded on the coverslips and allowed to spread for 2 hrs. Cells were next fixed in 4% paraformaldehyde for 20 min in PBS at room temperature and washed with PBS. Actin filaments (F-actin) were stained with Alexa 568-conjugated phalloidin (Molecular Probes, Eugene, OR) in PBS. The coverslips were mounted on object slides using Vectashield mounting medium containing 4',6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA). Fluorescence images were captured using a Nikon TE2000-E inverted microscope. Signals were quantified using the ImageJ software. Average values of 5 different images were plotted.

Plasmid construction, site-directed mutagenesis, and 3'UTR luciferase reporter analysis

The nucleotide sequence (~1,120 bp) covering the 3'UTR of the FERMT2 (Kindlin-2) gene was PCR-amplified from PC3 genomic DNA and subcloned into the pmirGlo vector downstream of the firefly luciferase expression cassette (Promega). The correct sequence and orientation were verified by sequencing. QuikChange site-directed mutagenesis kit (Stratagene) was used to generate a 3'UTR mutant where the seed sequence recognized by miR-138 was scrambled. pmirGlo reporter plasmids (1 µg total plasmid) were transfected with Lipofectamine 2000 (Invitrogen) into the specified cancer cells and then seeded in 12-well plates (3×10^4 cells per well). Cells were collected after 48 hours for assay using the Dual-Luciferase reporter assay system (Promega), as described previously (10;11). For cotransfection experiments, synthetic miRNA mimics or miR Negative Control (5 nM), were added to the transfection mix. All experiments were done in triplicate with data averaged from at least 3 independent experiments.

Oligonucleotide primer sequences

Oligonucleotide primers used for genomic PCR and site directed mutagenesis were from IDT (San Diego, CA) and are as follows: K2-3'UTR-Forward, 5'-AAC TTC TTT GCC TTAC CA-3'; K2-3'UTR-Reverse, 5'-TCT CCC TCG CAC CCT TTT G-3'; Mut-miR138-

Forward 5'-GTA TTG ACT AGA TTA ATC ACT GT-3'; Mut-miR138-Reverse: 5'-ACA GTG ATT AAT CTA GTC AAT AC-3'. The sequence in red represents the scrambled seed sequence for miR138.

Statistical analyses

The data are presented as means $\pm$ standard deviations of at least three independent experiments. The results were tested for significance using an unpaired Student's *t* test. A *p* value less than 0.05 was considered significant.

RESULTS

Loss of Kindlin-2 sensitizes prostate cancer cells to the docetaxel-induced apoptosis and cell death

A previously published study (9) had shown that reduction of K2 expression in cell lines derived from castration-resistant prostate cancer, including PC3 cells, are more sensitive to cisplatin-induced cell death. Docetaxel, however, is now the therapeutic agent of choice to treat patients with CRPC before they develop chemoresistance (9). We, therefore, sought to investigate the potential role of K2 in the sensitization of CRPC-derived PC3 cells to apoptosis and cell death when exposed to docetaxel. High expression levels of K2 in PC3 cells were previously reported (9). We confirmed this observation by comparing expression levels of K2 between PC3 and DU145, two CRPC cell lines, and LNCaP, an androgen-dependent cell line. We found K2 protein levels to be at least 6-times higher in PC3 and DU145 than in LNCaP cells (Fig. 1A). Next, by means of siRNA-mediated knockdown, we showed that K2 expression levels were efficiently suppressed in K2-knockdown cells (K2-KD), both at the protein level (Fig. 1B) and at the mRNA level (Fig. 1C). Treatment with docetaxel (Doc) had no effect on K2 expression levels, both in the non-targeting siRNA-transfected (NT) cells and the siRNA transfected K2-KD cells (Fig. 1B and 1C). Interestingly, when we measured Annexin V staining by flow cytometry, we found knockdown of K2 expression (K2-KD cells) enhanced cell apoptosis by more than 40% ($p < 0.05$), and when K2 knockdown was combined with docetaxel (K2-KD/Doc cells), apoptosis was further increased by ~60% ($p < 0.01$) when compared to the untreated, NT cells (Fig. 1D). Cell death, as measured by propidium iodide staining, was also increased by ~40% ($p < 0.01$) in the K2-KD cells and by more than 60% ($p < 0.01$) when K2 knockdown was combined with docetaxel treatment (Fig. 1E). Thus, suppression of K2 in chemoresistant PC3 cells sensitizes these cells to docetaxel-mediated apoptosis and cell death.

In order to confirm that the enhanced sensitization to docetaxel was specific to the loss of Kindlin-2 and not to an off target effect of the K2 siRNA, we used an siRNA that targets the 3'UTR of K2 (K2-KD-R) to knockdown endogenous Kindlin-2 and overexpressed a GFP-K2 fusion transcript lacking the K2 3'UTR and, therefore, insensitive to the knockdown effect of K2 3'UTR-targeting siRNA. Indeed, Figure 2A shows that the 3'UTR-targeted siRNA was very efficient in suppressing expression of endogenous K2, but had no apparent effect on our ability to express exogenous K2 (K2-KD-R). In contrast, the K2 ORF-targeted siRNA (K2-KD) inhibited expression of both endogenous K2 and the exogenous GFP-K2 (Fig. 1A). This strategy allowed us to assess the effect of restored K2 expression on

apoptosis in combination with docetaxel treatment. Docetaxel treatment of the control cells that were transfected with GFP and the non-targeting siRNA (GFP/NT cells) resulted ~50% increase in apoptosis when compared to the vehicle treated control cells ($p<0.05$), as measured by Annexin V staining levels (Fig. 2B and 2C). In addition, and consistent with the findings reported in Figure 1, docetaxel treatment of K2 knockdown (GFP/K2-KD/Doc) cells resulted in a further enhancement of apoptosis, which increased from 50% to 71% ($p<0.05$). On the other hand, when K2 expression was restored in the K2-depleted cells (K2/K2-KD), apoptosis, as indicated by Annexin V staining, was reduced to only 18% ($p<0.05$) after treatment with docetaxel (Fig. 2B and 2C). In fact, overexpression of exogenous K2 inhibited apoptosis to levels even lower than those observed in the control cells (Fig. 2B and 2C). Thus, our data strongly suggest that, while knockdown of K2 sensitizes cancer cells to docetaxel-induced apoptosis, overexpression of K2 confers resistance to apoptosis.

Combination of reduced Kindlin-2 and Docetaxel treatment has a dramatic inhibitory effect on integrin $\beta 1$ -mediated cell spreading

The integrin $\beta 1$ subunit is highly expressed in several cancer types including PCa and plays a key role in the pathology of these cancers (10;17;18). Moreover, integrins, in general, and $\beta 1$ integrin, in particular, has been linked to chemoresistance of tumor cells by promoting cell adhesion and spreading (19–22). Therefore, we considered that K2-mediated regulation of $\beta 1$ integrin might be involved in the modulation of chemosensitivity of prostate cancer cells. First, we showed that $\beta 1$ integrin is highly expressed on the surface of PC3 cells by flow cytometry (Fig 3 A). We also assessed expression levels of $\beta 3$ integrin, another well-established β integrin subunit implicated in cancer pathology and regulated by K2 (23–25), but found little to no expression on the surface to PC3 cells (Fig. 3B). Knockdown of K2 and treatment with docetaxel either alone or in combination did not alter surface expression levels of either $\beta 1$ or $\beta 3$ integrins (Fig 3B and 3C). We used cell spreading on fibronectin as readout of $\beta 1$ integrin function, since fibronectin is a well-established ligand for $\beta 1$ integrin, and cell spreading downstream of $\beta 1$ integrin signaling has been shown to promote chemoresistance (20–22). We assessed the effect of loss of K2 alone or in combination with docetaxel treatment (Fig 3D) on $\beta 1$ integrin-mediated cell spreading on fibronectin. When cells were seeded on glass coverslips in the absence of exogenous ligand (no fibronectin), they adhered to the surface but failed to spread and maintained a small, rounded shape (Fig. 3E). The same phenotype was observed with the cells that were treated with docetaxel in the absence of ligand (No ligand/Doc). However, when cells were seeded on fibronectin-coated coverslip glasses (NT cells), the cells began to spread and their area increased several fold compared to the cells on no ligand (Fig. 3E and 3F). Cell spreading was significantly ($p<0.05$) inhibited by knockdown of K2 (K2-KD, Fig. 3E). This inhibitory effect on cell spreading was further enhanced when the K2-knockdown cells were treated with docetaxel (K2-KD/Doc cells, $p<0.05$).

Overexpression of exogenous K2 in PC3 cells (NT/K2 cells, Fig. 4A) resulted in ~2-fold increase ($p<0.05$) in cells spreading (Fig 4B and 4C). Treatment of K2-overexpressing cells with docetaxel (NT/K2-Doc cells) inhibited cell spreading; this suppression did not reach the level observed in the PC3 cells expressing endogenous K2 only (NT/GFP and NT-GFP-

Doc cells). More importantly, overexpression of exogenous K2 in the K2-depleted cells (K2-KD/K2) not only restored cell spreading caused by K2-knockdown (K2-KD/GFP) but also negated the inhibitory effect of docetaxel on cell spreading (Fig. 4B and 4C).

miR-138 regulates Kindlin-2 expression and its modulation of docetaxel-mediated apoptosis

The role of microRNAs in the regulation of resistance to chemotherapy is well documented in the literature (26–30). To probe further in the underlying molecular and genetic mechanisms involved in K2-mediated modulation of the chemosensitivity of PC3 cells, we sought to investigate the role of microRNAs in the regulation of K2 expression. Our *in silico* analyses, using several microRNA prediction algorithms (Target Scan, miRanda and PITA) identified microRNA miR-138 as the top predicted microRNA to target K2 (Fig 5A). Further reinforcing our focus on miR-138 as a potential regulator of K2 are published findings that this microRNA is a modulator of chemosensitivity of other cancer types (26–30). As an initial step, we measured expression levels of miR-138 and found it to be very low in PC3 cells (Fig. 5B). In fact, miR-138 levels in the PC3 cells, which express high levels of K2, were more than 25-fold lower ($p<0.05$) than in LNCaP cells, which have very low levels of K2 (Fig. 5B).

Next, we showed that the seed sequence of miR-138 is a perfect match to a sequence in the 3'UTR of K2 (Fig. 5A). Then, using both qRT-PCR (Fig 5C) and western blot (Fig. 5D) analyses, we showed that transient transfection of PC3 cells with miR-138 mimics resulted in ~70% knockdown ($p<0.05$) of K2 transcript levels (Fig. 5C). The knockdown of K2 transcript via miR-138 was similar to that obtained with K2-specific siRNAs (K2-KD) while anti-miR-138 had no significant effect on K2 transcript levels. The miR-138-mediated knockdown of K2 was also confirmed by western blot analysis (Fig. 5D). Hence, the decrease in K2 expression levels is specific to miR-138. microRNAs regulate the expression of target genes by directly targeting and binding to specific seed sequences within the 3'UTR of the mRNAs, leading either to their degradation or to inhibition of their translation (31;32). To further confirm that miR-138 directly binds and represses expression of K2 transcripts, we used a luciferase gene reporter assay. PC3 cells, which express very low levels of miR-138, were co-transfected with the empty pmirGlo vector or the K2-3'UTR-pmirGlo construct along with either a non-targeting micro-RNA (NT-miR) or the miR-138 mimics (miR138). As expected, no difference in luciferase activity was found between the cells transfected with the control vector and the cells transfected with the vector containing the 3'-UTR of K2 in the presence of either the non-targeting microRNA or the anti-miR-138 (Fig. 5E). In contrast, overexpression of miR-138 mimics resulted in 70% reduction ($p<0.05$) in luciferase activity in the cells transfected with the K2 3'-UTR (Fig. 5E). Mutation of the seed sequences of miR-138 in the 3'-UTR of K2 abrogated the effect of the exogenous miR-138 in PC3 cells transfected with the K2-3'UTR- pmirGlo construct; the levels of luciferase activity did not show any significant difference when compared to the cells treated with the non-targeting microRNA (Fig. 5F). Thus, we confirmed that miR-138 specifically binds to a target sequence within the 3'UTR of K2 transcript and inhibits K2 expression.

Next, we tested whether the effects of K2 knockdown by siRNA on apoptosis and cell death were recapitulated by miR-138. Overexpression of miR-138 mimics in PC3 cells not only inhibited K2 expression to levels similar to those caused by K2-specific siRNA (Fig. 6A) but also induced apoptosis and cell death to levels similar to those induced by siRNA-mediated knockdown of K2 (Fig 6B and 6C, respectively). The combination of miR-138 and docetaxel treatment further enhanced apoptosis and cell death to levels similar to those induced by the K2 siRNA + docetaxel (Fig. 6B and 6C). Thus, by specifically targeting K2 and suppressing its expression, miR-138 sensitizes PC3 cells to apoptosis and cell death induced by docetaxel and mirrors the effect the siRNA-mediated down-regulation of K2.

Overexpression of a miR138-resistant variant of Kindlin-2 overcomes the inhibitory effect of miR-138 on apoptosis and cell adhesion

It is well established that a single microRNA is capable of targeting several different genes. Therefore, to confirm that the effect of miR-138 on apoptosis is the consequence of the specific targeting of K2 and not due to the broad effects of miR-138 on its target genes, we overexpressed a miR-138-resistant variant of K2 (a K2 construct that lacks the 3'UTR) in miR-138 overexpressing PC3 cells (Fig. 7A), and assessed sensitivity to chemotherapy. Introduction of the miR-138-resistant variant of K2 was sufficient to inhibit apoptosis that is induced by miR-138 + docetaxel-treatment (Fig. 7B and 7C). In fact, and as in the case of K2 overexpression in siRNA K2-depleted cells, restoration of K2 expression in the miR-138-expressing cells resulted in a 3-fold decrease ($p<0.05$) in apoptosis compared to the miR-138-expressing cells alone or the K2-knockdown cells also (K2/miR138/Doc and K2/K2-KD/Doc cells versus GFP/miR138/Doc and GFP/K2-KD/Doc cells). Thus, overexpression of K2 in miR-138-expressing cells was not only able to overcome the effect of miR-138 in inducing apoptosis, but was also sufficient to confer greater resistance to the apoptotic effect of docetaxel. Therefore, we confirmed that the K2-mediated modulation of chemosensitivity in PC3 cells is specifically regulated downstream of miR-138.

Lastly, we verified that overexpression of the miR-138-resistant variant of K2 in the miR-138-expressing cells (miR138/K2 and miR138/K2-Doc cells) was sufficient to restore cell spreading that was inhibited by miR-138 and miR-138 + docetaxel combination (Fig. 7D and 7E). In fact, overexpression of K2 resulted in ~2-fold increase in cell spreading ($p<0.05$) when compared to the GFP-expressing cells under the same treatment conditions (NT/GFP and NT/GFP-Doc cells versus miR138/K2 and miR138-K2-Doc cells, respectively). Thus, while miR-138 inhibits cell spreading, overexpression of K2 is sufficient to overcome the inhibitory effect of miR-138. Finally, taken together, our data we identify the miR-138 $\leftrightarrow$ K2 $\leftrightarrow$ β 1 integrin signaling axis as a major player in the modulation of chemosensitivity in prostate cancer cells.

DISCUSSION

Cancer metastasis is responsible for approximately 90% of the cancer deaths, including PCa patients. Androgen is the main driver of the growth and proliferation of PCa tumors; and, therefore, androgen ablation by means of hormonal therapy and prostate castration is used as the primary strategy to limit disease progression. mCRPC often develops as a result of

unresponsiveness of the primary tumor to the initial hormonal therapy. The main treatment option then becomes a docetaxel regimen, which has been shown to extend the lives of patients with mCRPC until they start developing chemoresistance. Therefore, identifying ways to diminish chemoresistance represents a viable strategy to increase the efficacy of chemotherapy and to limit the systemic cytotoxicity often associated with chemotherapeutics. In case of mCRPC, patients initially respond well to docetaxel, but eventually develop resistance. Although several factors can be attributed to resistance to docetaxel treatments (33), the molecular mechanisms underlying chemoresistance still remain poorly understood. In the present study, we sought to investigate a novel mechanism whereby inhibition of Kindlin-2 in combination with docetaxel might significantly enhance the sensitivity of PCa cells to apoptosis and cell death and therefore inhibit disease progression.

We applied a combination of genetic and pharmacological manipulations, as well as different biochemical and cell imaging assays, to investigate the role K2 in the modulation of the docetaxel-mediated apoptosis and cell death in PCa cells. We showed that (i) K2 is overexpressed in the PCa cell lines derived from mCRPC tumors (DU148 and PC3) compared to cell derived from androgen-dependent tumors (LNCaP); (ii) while knockdown of K2 expression in PC3 cells significantly enhanced response to docetaxel, over-expression of K2 rendered PC3 cell more resistant to apoptosis and cell death in response to docetaxel, suggesting that K2 may play a critical role in the modulation of chemosensitivity; (iii) the K2-mediated sensitization to docetaxel treatment is accompanied by inhibition of $\beta 1$ integrin signaling; and (iv) microRNA miR-138 targets K2 to regulate its expression, and therefore its modulation of chemosensitivity.

Mechanistic considerations

Our data show on one hand that treatment with docetaxel has no effect of K2 expression levels. On the other hand, sensitization of PC3 cells to chemotherapeutics results from the specific loss of K2 expression and not due to off-target effect of the siRNA, since overexpression of exogenous K2 was sufficient to overcome the effect of the K2 knockdown and to confer greater resistance to docetaxel.

The K2/Integrins link to sensitization to chemotherapeutics—The involvement of integrins in general and $\beta 1$ integrin signaling in particular in the modulation of chemosensitivity of tumors is widely documented (20–22;34–37). In particular, several studies have shown that $\beta 1$ integrin signaling, by promoting cancer cell spreading and aggregation was responsible for enhancing resistance to chemotherapeutics (20–22). We demonstrated the link between K2 and $\beta 1$ integrin signaling and chemosensitivity when we assessed the effect of K2 siRNA-knockdown on $\beta 1$ integrin activity and found that, while knockdown of K2 expression inhibits $\beta 1$ integrin activity, overexpression of exogenous K2 restores and even enhances $\beta 1$ integrin-mediated cell spreading. We therefore identified Kindlin-2 as a critical regulator of $\beta 1$ integrin in the regulation of cell spreading and chemosensitivity. Integrins other than the $\beta 1$ subunits have been shown to be expressed in PCa cell lines (38), and, may be involved in the modulation of chemoresistance in PCa. Our study, however, focused on $\beta 1$ -integrin subunit based on its levels of expression in PCa

compared to other integrin subunits, and on the wide literature documentation on its involvement in chemoresistance in several cancer types (20–22;34–37). Our data have shown that the chemotherapeutic-mediated cell death in the K2-knockdown cells is caused by apoptosis as demonstrated by increased Annexin V staining. However, cell death can also be induced by anoikis after detachment of cells from the extracellular matrix as a result of K2-knockdown-mediated disruption of integrin signaling. Therefore, we are not excluding anoikis as an additional mechanism for the therapeutics-mediated cell death.

The miR138/K2/ β 1 integrin signaling axis—microRNAs have been established as powerful regulators of gene expression under normal physiological as well as pathological conditions, including cancer progression and metastasis and in the regulation of chemoresistance. Recent studies have demonstrated a key role of miR-138 in the progression and metastasis (39) and the development of chemoresistance in cancers of different origins (29;40). We found miR-138 to specifically target K2 and to suppress its expression. Interestingly, expression of miR-138 in mCRPC PC3 cell line is ~25-fold lower than in the androgen sensitive LNCaP cells (Fig. 5B). In fact, the miR138/K2 transcript ratio is more than 100-fold low in PC3 cells, which is consistent with the high levels of K2 transcript and protein in these cells. More importantly, overexpression of miR-138 mimics in PC3 cells specifically inhibits K2 expression and its downstream effect on sensitizing PC3 cells to the docetaxel-induced apoptosis and cell death. This miR-138 effect is negated when a miR-138-resistant variant of K2 is introduced into PC3 cells at the same time as the miR-138 mimics, therefore demonstrating the specificity of miR-138 in targeting K2.

In conclusion, we have identified a novel signaling axis, $\text{miR-138} \leftrightarrow \text{K2} \leftrightarrow \beta 1$, where K2 plays a critical role in the signaling mechanisms that modulate chemosensitivity (Fig. 7F). Our study therefore provides the underpinnings of a new therapeutic strategy targeting K2 to be used in combination with chemotherapy for patients with mCRPC. Extending our investigations to other cell lines, human biopsy samples and mice xenograft models are next logical steps of future studies to bring K2 targeting closer to implementation.

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List of Abbreviations

K2	Kindlin-2
PCa	Prostate Cancer
mCRPC	Metastatic castration resistant prostate cancer
cDNA	Complementary-DNA
AR	Androgen receptor
Doc	Docetaxel

GFP	Green fluorescent proteinsiRNA, small interfering RNA
qRT-PCR	Quantitative Reverse Transcriptase PCR

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IMPLICATIONS

Targeted inhibition of Kindlin-2 in combination with chemotherapy represents an effective treatment option for mCRPC.

Figure 1. Knockdown of Kindlin-2 expression sensitizes mCRPC PC3 cells to the docetaxel-induced apoptosis and cell death

(A) Western blots of cell lysates from LNCaP, DU145 and PC3 cells with anti-Kindlin-2 antibody. The numbers under the bands represent the fold change in signal intensity after normalization to the signal from LNCaP cells. β -Actin was used as an internal control. (B) Western blots of cell lysates from PC3 cells with anti-Kindlin-2 antibody after the indicated treatments: NT, non-targeting siRNA; K2-KD, Kindlin-2 knockdown with K2 siRNA. β -Actin was used as an internal control. (C) Quantification of Kindlin-2 transcript using qt-RT-PCR in PC3 cells under the indicated treatments. (D & E) Quantification of apoptosis (D) and cell death (E) in PC3 cells after staining by Annexin V for apoptosis, and Propidium Iodide for cell death. Data are the fold-change in apoptosis or cell death normalized to the values found in their control cells transfected with GFP and the non-targeting siRNA. Data are representative of 3 independent experiments (*, $p < 0.05$; Student's t-test).

Figure 2. Over-expression of a Kindlin-2 knockdown-resistant variant restores resistance to apoptosis induced by docetaxel

(A) Western blots of cell lysates from PC3 cells with the indicated transfections with anti-Kindlin-2 antibody (Top panel) and anti-GFP antibody (Middle panel). K2-KD-R represents knockdown of Kindlin-2 with an siRNA that targets the 3'UTR of Kindlin-2. The Kindlin-2 band in the GFP panel is the result of uncompleted stripping of the membrane after being probed with anti-K-2 antibody. Note that both antibodies were raised in mice. β -Actin was used as a loading control. (B) Representative histograms using flow cytometry of PC3 cells with the indicated transfections after docetaxel treatment and staining for Annexin V as an apoptosis marker. Numbers in parenthesis represent the % change in apoptosis compared to the untreated cells. (C) Quantitation of apoptosis in PC3 cells. Data are the fold-change in cell apoptosis normalized to the values found in their control cells transfected with GFP and the non-targeting siRNA (GFP/NT) cells. Data are representative of 3 independent experiments (*, $p < 0.05$; Student's t-test).

Figure 3. Combination of Kindlin-2 knockdown and docetaxel treatment has a dramatic inhibitory effect on integrin β 1-mediated cell spreading
 (A, B & C) Representative histograms using flow cytometry of PC3 cells with the indicated transfections and staining with PE-conjugated anti CD29 antibody for cell surface β 1 integrin (A & B) and with FITC-conjugated anti CD61 antibody for β 3 integrin (C). (D) Western blots with anti-K2 antibody of cell lysates from PC3 cells with the indicated transfections. β -Actin was used as a loading control. (E) Representative micrographs of Alexa 568 phalloidin-stained PC3 cells that were transfected with either a non-targeting siRNA (NT) or Kindlin-2 siRNA and seeded on uncoated coverslips (No Ligand) or on coverslips coated with fibronectin (20 μ g/ml) for 2 hrs in the presence or absence of docetaxel (Doc). (F) Quantification of PC3 cell spreading under the indicated treatments. Data are the fold-change in cell spreading normalized to the values for untreated and non-targeting siRNA transfected cells. Data are representative of 3 independent experiments (*, $p < 0.05$; Student's t-test).

Figure 4. Over-expression of a K2 knockdown-resistant variant overcomes the combined K2 knockdown/docetaxel inhibitory effect on cell spreading

(A) Western blots of cell lysates from PC3 cells with the indicated transfections with anti-Kindlin-2 antibody (Top panel) and anti-GFP antibody (Middle panel). K2-KD-R represents knockdown of Kindlin-2 with an siRNA that targets the 3'UTR of Kindlin-2. β -Actin was used as a loading control. (B) Representative photomicrographs of Alexa 568 phalloidin-stained PC3 cells that were transfected as indicated and seeded on coverslips coated with Fibronectin (20 μ g/ml) for 2 hrs, in the presence or absence of docetaxel. (C) Quantification of PC3 cell spreading under the indicated treatments. Data are the fold-change in cell spreading normalized to the values found in the untreated and non-targeting siRNA transfected cells. Data are representative of 3 independent experiments (*, $p < 0.05$; Student's t-test).

Figure 5. miR-138 regulates Kindlin-2 expression

(A) Schematic representation FERMT2 (Kindlin-2) transcript showing the location of the seed sequence of microRNAs miR-138 within the 3'-UTR. The nucleotide sequence and location of the seed sequence with respect to the 3'-UTR is shown, as well as its alignment with the miR-138 sequence. (B) Quantification of miR-138 levels in PC3 cells using qt-RT-PCR. Values were normalized to LNCaP cells. (C) Quantification of Kindlin-2 transcript levels in PC3 cells under the indicated treatments. Values were normalized to the untreated cells. (D) Western blots of cell lysates from PC3 cells with the indicated transfections with anti-Kindlin-2. β-Actin serves as a loading control. (E) Firefly luciferase reporter plasmid pmirGlo containing the 3'-UTR of Kindlin-2 was transiently transfected into PC3 cells and treated as indicated. Luciferase activities were measured after 48 h and plotted after being normalized to the non-targeting microRNA-transfected cells. in (F) the same experiment was

performed with the K2-3'UTR where the seed sequence of miR-138 was scrambled. Data are representative of 3 independent experiments (*, $p < 0.05$; Student's t-test).

Figure 6. miR-138 regulates the Kindlin-2-mediated modulation of docetaxel-mediated sensitization to apoptosis
 (A) Western blots with anti-Kindlin-2 antibody of cell lysates from PC3 cells with the indicated transfections. β -Actin is a loading control. (B & C) Quantification of apoptosis (B) and cell death (C) in PC3 cells under the indicated treatments. Data are the fold-change in cell apoptosis normalized to the values found in the control cells transfected with the non-targeting siRNA. Data are representative of 3 independent experiments (*, $p < 0.05$; Student's t-test).

Figure 7. Over-expression of a miR138-resistant variant of Kindlin-2 overcomes the inhibitory effect of miR138 on apoptosis and cell spreading induced by loss of Kindlin-2 and docetaxel treatment

(A) Western blots with anti-Kindlin-2 antibody (Top panel) and anti-GFP antibody (Middle panel) of cell lysates from PC3 cells with the indicated transfections. β -Actin served as a loading control. (B) Representative histograms using flow cytometry of PC3 cells with the indicated transfections after docetaxel treatment and staining with Annexin V for apoptosis. Numbers in parenthesis represent the % change in apoptosis compared to the untreated cells. (C) Quantification of apoptosis in PC3 cells. Data are the fold-change in cell apoptosis normalized to the values of control cells transfected with GFP and the non-targeting siRNA (GFP/NT) cells. Data are representative of 3 independent experiments (*, $p < 0.05$; Student's t-test). (D) Representative micrographs of Alexa 568 phalloidin-stained PC3 cells with the indicated transfections and seeded onto coverslips coated with fibronectin (20 μ g/ml) for 2 hrs in the presence or absence of docetaxel. (E) Quantification of PC3 cell spreading under the indicated treatments. Data are the fold-change in cell spreading normalized to the values of untreated cells transfected with GFP and the non-targeting siRNA (GFP/NT). Data are representative of 3 independent experiments (*, $p < 0.05$; Student's t-test). (F) Schematic diagram illustrating the cross-talk between miR-138, Kindlin-2 and β 1-Integrin to dictate the modulation of response to chemotherapeutics.