KDM5B expression in cisplatin resistant neuroblastoma cell lines

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Received March 14, 2022; Accepted July 21, 2022

DOI: 10.3892/ol.2022.13485

Abstract. Chemoresistance is a major problem in successful cancer therapy. Lysine-specific demethylase 5B (KDM5B), is a member of the KDM5 family of histone demethylases, whose dysregulation has been observed in numerous types of cancer and plays a role in drug tolerance. The present study examined KDM5B expression in high risk neuroblastoma cell lines. Its level was markedly reduced in cisplatin-resistant cells, UKF-NB-4^CDDP, compared with parental sensitive cells UKF-NB-4. Moreover, KDM5B-silencing did not affect either viability nor the response to CDDP in resistant cells, and led to increase of proliferation and migration in CDDP resistant cells but not in sensitive ones. Compliant with these results, short interfering KDM5B transfection resulted in increased S phase in resistant cells. Overall, these findings suggested that KDM5B may be involved in the survival mechanisms of neuroblastoma cells, which makes KDM5B a promising factor for the prediction of sensitivity to CDDP that should therefore be considered for future research.

Introduction

Epigenetic mechanisms are essential for the normal development and maintenance of cell and tissue-specific gene expression patterns in mammals (1). DNA methylation, histone modification, nucleosome remodeling, and RNA-mediated targeting regulate numerous biological processes that are fundamental to the genesis of cancer. Disruption of epigenetic processes can lead to altered gene function that can induce malignant cell transformation (2). Histone methylation plays an important role in the regulation of genes expression, its dysregulation has been observed in various cancers (3-7). Regulation of methylation is mediated by two types of enzymes-histone methyltransferases, which add methyl groups to arginine and lysine residues, and histone demethylases (lysine demethylase-KDM), which remove methyl groups (3). The KDM5 family of lysine demethylases known also as Jumonji C (JmjC) or JARID1 that have four members (KDM5A-D), demethylate di- and tri-methylated H3K4 (8). These enzymes are 2-oxoglutarate-dependent dioxygenases that require for their function Fe^2+ and oxygen in order to undergo the hydroxylation necessary to remove the methyl groups (8). KDM5B, also known as JARID1B, has been found to associate with transcription factors PAX9, FOXC2 and FOXG1. It can also repress or promote activation of target genes by interacting with nuclear hormonal receptors (9). The levels of enzyme modifying histones KDM5B determine the hyperactivation of PI3K/AKT signaling in prostate cancer (10). Dysregulation of KDM5B has been identified in numerous cancers e.g. laryngeal squamous cell carcinoma, bladder, breast cancer, and is closely correlated with tumorigenesis, metastasis, and worse survival in humans (5-7). Therefore, this enzyme might be a potential promising target for novel cancer diagnostic and/or treatment.

KDM5B has been described as important for the formation and maintenance of cancer stem cells in neuroblastoma cell lines (NBL) (11). In addition, its overexpression was a marker of shorter relapse-free survival in patients with NBL (11). NBL is a malignant embryonal tumor in children, emerging from the peripheral nervous system. The biology of NBL is heterogeneous; small groups of NBL regress spontaneously, while numerous cases have aggressive behavior. For high-risk neuroblastoma (HR-NBL) is characteristic development of chemoresistance (12). Patients suffering from HR-NBL have a 5-year overall survival rate of ~40% despite all intensive multimodal therapies. To date, there are no salvage treatment regimens known to be curative (12,13). Knowledge of MYCN properties is limited because its expression is in physiological conditions limited to the early stages of embryonic development (14). N-myc protein interacts with Max and its high levels, which occurs in MYCN amplified NBL, lead to a large number of transcription-activating complexes (15). MYCN overexpression induces proliferation and suppresses apoptosis and differentiation in NBL cells (16). Several studies proved that MYCN silencing in MYCN amplified NBL cells suppressed growth and induced apoptosis and differentiation e.g. (14,16,17). Its expression was higher in MYCN amplified NBL cell lines than in MYCN-non-amplified NBL cells (11).

MYCN amplification correlates with poor outcome of NBL patients. Examination of MYCN amplification is part of diagnostic scheme in NBL. MYCN is MYC family of transcription
factors member. Those transcription factors are regulators of cellular proliferation, differentiation and survival (12,13).

The aim of this study is to investigate the importance of KDM5 expression for the growth of NBL cells and their chemoresistance to cisplatin [CDDP abbreviation of cis-diaminedichloroplatinum (II)].

Materials and methods

Cell culture and chemicals. Human HR-NBL cell lines UKF-NB-4, UKF-NB-4-CDDP, SK-N-AS, SK-N-AS CDDP/UKF-NB-3 and UKF-NB-3 CDDP were donated by prof. J. Cinatl, Dr. Sc. From Goethe University in Frankfurt am Main. Cells were grown in Iscove’s Modified Dulbecco’s medium (IMDM) supplemented with 10% (v/v) fetal bovine serum (both Thermo Fisher Scientific) and incubated at 37°C in 5% CO₂. For experiments, 8x10⁵ cells were seeded in 22.1 cm² dishes and after 24 h treated with cisplatin (Ebewe) in final concentration 20 µM for 48 h. Both cell lines amplified MYCN gene as we proved by FISH (data not shown).

Assessment of cisplatin cytotoxicity. To evaluate CDDP cytotoxicity, MTT (3-(4,5-dimethylthiazol-2-yl)-2.5 diphenyltetrazolium bromide) assay was performed. 10⁴ cells/well were seeded in 96-well cell culture plate and cells were treated with CDDP at final concentration 0.6-300 µM for 48 h. Subsequently, MTT solution (2 mg/ml in PBS) (Fluka) was added and the plate was placed in an incubator for 2 h. Cells were then lysed in solution of 20% SDS (Invitrogen) containing 50% N,N-dimethylformamide (Sigma-Aldrich), pH 4.5, and the absorbance at 570 nm was measured by multiwell ELISA reader Versamax (Molecular Devices). The optical density of the medium was read as background and the optical density value of the live control cells was taken as 100%. The values of IC₅₀ were determined using at least 3 independent measurements by SOFTmaxPro software.

Transfection. NB cells were transfected with a smart pool siRNA to KDM5B ON-TARGETplus Human KDM5B siRNA, cat. No. L-009899-00-0020 (https://horizondiscovery.com/en/search?searchterm=L‑009899‑00‑0020) and Lincode Non-targeting siRNA, cat. No. L-009899-00-0020 (https://horizondiscovery.com/en/search?searchterm=L‑009899‑00‑0020) and Lincode Non-targeting siRNA Lincode Non-targeting siRNA #1, cat. No. D-001320-01-20 (https://horizondiscovery.com/en/search?searchterm=D‑001320‑01‑20+) using Dharmafect transfection reagent (all purchased from Dharmacon) according to the manufacturer's instructions. The siRNA concentration was 25 nM.

RNA isolation and quantitative RT-PCR. RNA was isolated using PureLink RNA Mini Kit (Thermo Fisher Scientific) according to the manufacturer's protocol. Quantity and quality were verified using the NanoDrop One spectrophotometer (Thermo Fisher Scientific). Reverse transcription was performed using qRT-PCR Kit (Generi Biotech) and 1,000 ng of RNA was used for complementary DNA synthesis. Primers and probes hKDM5B_Q1 and POLR2A that was used as an endogenous control (18), were designed and produced by Generi Biotech. Custom oligo synthesis, cat. No. 1000-020 for gene: KDM5B; Gene ID: 10765 POLR2A; Gene ID: 5430 (https://www.generi-biotech.com/products/custom-oligo-synthesis/). We used POLR2A as an internal standard because it is homogeneously and uniformly expressed in NBL cells (18). It is also used by other groups studying NBL (19,20).

The quantification of gene expression was performed using Quantstudio 3 Real-Time PCR System (Thermo Fisher Scientific) in triplicate. The temperature profile was: 95°C for 3 min, 50 cycles of 95°C for 10 sec, 60°C 20 sec. Fold change values were determined using REST 2009 software.

Western blot analysis. Proteins were extracted in RIPA Buffer supplemented with Complete protease inhibitor cocktail (Roche) and their concentration was measured by DC protein assay (Bio-Rad Laboratories). Samples (40 µg) were resolved on SDS polyacrylamide gels and blotted on nitrocellulose membranes (Bio-Rad). Primary antibody JARID1B Rabbit mAb Cell Signaling Technology was diluted 1:1,000, β-actin Mouse mAb (Sigma-Aldrich) diluted 1:3,000 was used as a loading control. Secondary antibodies Europium conjugated anti-IgG (Molecular Devices) were diluted 1:5,000. Membranes were visualized by SpectraMax i3x Multi-Mode Microplate Reader (Molecular Devices). ImageJ 1.52a software was employed for the analysis.

Cell viability assay. Cells were seeded in 24-well cell culture plate at a density of 4x10⁴ cells/well and incubated with PrestoBlue Cell Viability Reagent (Thermo Fisher Scientific) for 30 min at 37°C. The fluorescence was measured using an excitation wavelength of 560 nm and emission of 590 nm by SpectraMax i3x Multi-Mode Microplate Reader (Molecular Devices). Each sample was analyzed in triplicate.

Cell proliferation. Cells after transfection were seeded into 16-well E-plates for impedance-based detection (ACEA Bioscience Inc) at density of 10⁵ cells per well. The xCELLigence RTCA DP Instrument (ACEA Bioscience Inc) placed in a humidified incubator at 37°C and 5% CO₂ was used for real-time monitoring of cell proliferation. The cell index was monitored every 30 min for 85 h and data were recorded by the supplied RTCA software. Each sample was analyzed in triplicate.

Determination of histone H3K4 methylation status and KDM5B level. Flow cytometry was used for the detection of H3K4 trimethylation and expression of KDM5B on protein level. Cells after treatment and/or transfection were washed with cold PBS (Thermo Fisher Scientific), trypsinized (trypsin-Thermo Fisher Scientific) and collected by centrifugation. Pellets of cells were washed with PBS and fixed in 3.6% paraformaldehyde for 15 min at room temperature. Cell pellets were then washed with PBS and permeabilized by 90% methanol for 1 h at -20°C. Pellets were subsequently washed 3 times with 0.5% bovine serum albumin (BSA-Roth) in PBS and were resuspended in primary antibody JARID1B Rabbit mAB diluted 1:1,000 (Cell Signaling Technology) or Anti-trimethyl-Histone H3 (Lys4) Rabbit (EMD Millipore Corp.) at dilution 1:400 and incubated for 1 h at laboratory temperature. Cells were then washed with 0.5% BSA, resuspended in fluorochrome-conjugated secondary antibody Anti-Rabbit IgG (H+L) Alexa Fluor® 647 Conjugate (Thermo Fisher Scientific) diluted 1:500 and incubated for 30 min at room temperature in dark. Washed and re-suspended cells were
measured using a BD FACSCelesta (BD Bioscience), and data were analyzed by Flowlogic software (Inivai Technologies).

Cell cycle analysis. Cells after treatment and/or transfection were washed with cold PBS, trypsinized and collected by centrifugation. Pellets of cells were washed with PBS and fixed in 3.6% paraformaldehyde for 10 min at room temperature. Cell pellets were then washed with PBS and permeabilized by 90% methanol for 1 h at -20°C. Pellets were washed with PBS, resuspended in 500 µl PBS and one drop of FixCycle™ Violet Ready Flow™ Reagent (Thermo Fisher Scientific) was added and after 30 min incubation were cells measured using a BD FACSCelesta (BD Bioscience), and data were analyzed by Flowlogic software (Inivai Technologies).

Wound healing assay. Neuroblastoma cells were seeded in 9.2 cm² dish in number 1.6x10⁶ cells/ml of sensitive cells and 2.2x10⁵ cells/ml of resistant cells, that allowed to reach 70% confluence for 24 h at 37°C in 5% CO₂ and then transfected with siRNA to KDM5B and Lincode non-targeting siRNA. 48 h after transfection was drawn the line across the dish's surface using a 1,000 µl sterile plastic tip, at that time the confluence was more than 80%. After wounding, cells were grown in Iscove's Modified Dulbecco’s medium (IMDM) with 5% (v/v) FBS (both Thermo Fisher Scientific) and incubated at 37°C in 5% CO₂. For scratch assay, 80-90% confluence is recommended so that the cells do not overgrow (21, 22). Pictures were captured at the same field immediately, 24 and 48 h after the wounding by microscope Olympus IX71 (Olympus) and ImageJ 1.52a software was employed for the analysis.

Statistical analysis. All experiments were independently repeated at least three times and data are shown as averages ± standard error. One-way Anova with post-hoc Tukey HSD and two-way ANOVA followed by Bonferroni test (https://astatsa.com/OneWay_Anova_with_TukeyHSD/) were utilized when comparing the situations. Results from RT-qPCR were statistically compared using REST 2009 software (23). Significances (P<0.05 was considered as significant) of the statistical analyses are shown in individual Figures and described in their legends.

Results

KDM5B is downregulated in resistant neuroblastoma cell line. UKF-NB-4⁴CDDP resulting from long-term cultivation with an increasing dose of CDDP was used as a model of drug resistance (24,25). We used this wide concentration range only to determine IC₅₀ using MTT test to demonstrate lower sensitivity in the cell line with experimentally induced chemoresistance (UKF-NB-4⁴CDDP) compared to sensitive cells (UKF-NB-4). We used only one concentration (20 mikroM) in further experiments. This cell line has ~4 times higher IC₅₀ compared to the parental line UKF-NB-4 (Fig. 1A). We examined the level of KDM5B mRNA and protein in both cell lines and the expression of this gene in UKF-NB-4⁴CDDP and in both lines after incubation with cisplatin was related to the expression in UKF-NB-4 control. QRT-PCR results showed that KDM5B expression was noticeably lower in resistant cell line (P<0.01). The same result was observed after incubation of these cells with CDDP; however, this compound did not further alter expression in resistant cell line (Fig. 1B). A decrease in the level of KDM5B expression was also observed in UKF-NB-4⁴CDDP at the protein level (Fig. 1C, D). Furthermore, we observed the same results in another NBL cell line UKF-NB-3, where UKF-NB-3⁴CDDP had lower levels of KDM5B mRNA (P<0.05) and protein. CDDP did not modulate KDM5B expression (Fig. S1). In SK-N-AS KDM5B level was decreased by 48 h incubation with CDDP. In SK-N-AS⁴CDDP, KDM5B was not modulated by cisplatin and there was no significant difference between sensitive SK-N-AS and resistant SK-N-AS⁴CDDP (Fig. S2).

KDM5B knockdown reduced KDM5B expression and upregulated histone H3K4 trimethylation in neuroblastoma cells. UKF-NB-4 and UKF-NB-4⁴CDDP cells were transfected with KDM5B siRNA for 48 h and transfection resulted in a significant suppression of KDM5B level compared to cells transfected with non-coding siRNA transfected cells (P<0.001) (Fig. 2A). Flow cytometry was performed to determine the level of KDM5B protein, which decreased in both siRNA transfected cell lines (P<0.01), while the trimethylation of histone H3K4me3 was significantly increased compared to the control group in UKF-NB-4 (P<0.01) and UKF-NB-4⁴CDDP (P<0.05) (Fig. 2B, C). The results demonstrated that KDM5B siRNA reduced KDM5B mRNA and protein expression and elevated protein H3K4me3 increased the trimethylation of histone H3K4 in UKF-NB-4 and UKF-NB-4⁴CDDP cell lines.

KDM5B knockdown promoted cell proliferation and migration in resistant cell line. Proliferation of neuroblastoma cells after KDM5B siRNA transfection was evaluated by xCELLigence system. We found that KDM5B knockdown inhibited cell proliferation in sensitive cell line; however, silencing of KDM5B in resistant cells led to increased proliferation (Fig. 3A). The wound healing assay showed that down-regulation of KDM5B promoted the migration of UKF-NB-4⁴CDDP cells compared to UKF-NB-4 (Fig. 3B). We also performed a cell viability assay, to see the impact of transfection on neuroblastoma cell lines. Results show, that KDM5B siRNA reduced the number of viable cells compared with non-coding siRNA transfected cells in sensitive cell line more significantly (P<0.01), than in resistant cell line (P<0.05). Increased sensitivity to CDDP (48 h treatment of these cells with CDDP) after silencing of KDM5B in sensitive cell line was observed (P<0.05). Interestingly, KDM5B knockdown affected neither viability nor response to CDDP in resistant cells (Fig. 3C).

KDM5B knockdown increases cell cycle S phase in resistant cell line. As shown above, KDM5B downregulation promotes cell proliferation and migration in resistant NBL cells (Fig. 3). Thus, we explored the role of KDM5B in cell cycle, using flow cytometry. Consistent with proliferation and migration data, we found that KDM5B knockdown resulted in a significant increase in the S phase in UKF-NB-4⁴CDDP resistant cell line (P<0.05). In the sensitive cell line UKF-NB-4, silencing did not lead to any significant change in cell cycle (Fig. 4).
Aberrant epigenetic modifications, such as histone methylation, are widely described as essential players in cancer development and progression (3,26). KDM5B, a histone lysine demethylase, whose dysregulation has been observed in numerous types of cancers and also has a role in the appearance of a drug-tolerant population (11,27,28). Growing evidence indicates that KDM5B act as an oncogene in numerous types of cancer, such as bladder, breast, lung, prostate, and ovarian cancer, and also in NBL (29-35). Since the development of chemoresistance in high-risk NBL is a
negative prognostic marker, we decided to investigate the importance of KDM5B expression for NBL cell growth and its chemoresistance to CDDP that is used in high-risk NBL therapy.
In this study, we demonstrated that KDM5B expression is markedly reduced in NBL cisplatin chemoresistant cells, compared to parental sensitive cells (Fig. 1) which is associated with enhanced cell migration and invasion, as well as it may be possibly involved in drug resistance (Fig. 3). However, KDM5B silencing did not change the sensitivity of resistant cells to CDDP. Several different mechanisms in chemoresistance that were described in several studies may be involved and KDM5B expression is only one of those mechanisms. For example in (25) we described several different mechanisms of chemoresistance in one cell line with experimentally induced chemoresistance to ellipticine. These findings are consistent with the reported data, showing that KDM5B can play a dual role in cancer (36). Roesch et al. found that KDM5B expression in malignant melanoma, especially in advanced and metastatic melanoma tissues, was significantly downregulated and this lysine demethylase has been shown to have immediate antiproliferative effects, but later has a role in continuous tumor growth and maintenance (37). Furthermore, the elimination of KDM5B leads to an initial acceleration of melanoma growth (28). The MYCN plays a crucial role in the malignant behavior of NBL and is associated with a poor prognosis (13,38). We detected a decrease in the level of KDM5B in UKF-NB-4CDDP and UKF-NB-3CDDP cisplatin resistant cell lines that are MYCN amplified while in SK-N-ASCDDP cell line without MYCN amplification KDM5B level has not been changed compared to sensitive SK-N-AS cells. We suppose that this may be caused by increased expression of MYCN in lines with amplification of this gene (39). Zhang et al. suggested that n-Myc represses KDM5B gene transcription by direct binding to the Spl-binding site-enriched region of the KDM5B gene promoter, most likely through the recruitment of histone deacetylases (40). This work showed that the suppression of KDM5B expression reduces NBL cell proliferation. However, n-Myc induces the proliferation of NBL cells and represses KDM5B expression, suggesting that n-Myc-mediated transcriptional repression of KDM5B counterintuitively reduces tumor cell proliferation (30).

In conclusion, the results of this study show that KDM5B knockdown leads to increased levels of H3K4me in both cisplatin sensitive and resistant cell lines (Fig. 2). Based on this finding, it can be concluded that the function of lysine demethylase KDM5B i.e. demethylation of di- and tri-methylated histone H3K4 cannot be fully replaced by the other KDM5 family members in NBL cells. We proved increased H3K4 me 3 also after silencing of KDM5D (Podhorska N. unpublished results) and KDM5A and C we did not test. We supposed that all KDM5 isoforms are necessary to ensure the adequate level of H3K4 me3. It can be concluded that the function of this histone lysine demethylase cannot be fully replaced by the others KDM5. Also, KDM5B silencing led to an increase of proliferation, and wound healing assay showed an increase in migration in resistant cell line. Moreover, in chemoresistant cells, it only minimally decreased viability after cisplatin treatment compared to sensitive cells (Fig. 3). Compliant with these results in resistant cells, siKDM5B transfection resulted in an increase in cell cycle S phase (Fig. 4). The effect of KDM5B on cell proliferation and the cell cycle of tumor cells varies in different tumors. The mechanism of the relationship between KDM5B and the cell cycle is not yet known, PI3K-AKT pathway activation (41), BRCA1 (42) and transcription factors E2F1 and E2F2 (43) are expected to be affected, but other mechanisms are also possible. In a series of tumors, its silence inhibits and reduces the percentage of cells in S phase, for example in prostate cancer (44), hepatocellular carcinoma (43), bladder cancer and small cell lung carcinoma (33) or acute lymphoblastic leukemia (42). On the other hand, in melanoma it has the opposite effect, i.e. the silencing of KDM5B accelerates growth and increases the proportion of cells in the S phase (37). KDM5B transfection induced cell differentiation in hypopharyngeal squamous cell carcinoma and, on the contrary, it’s silencing accelerated growth of cells (41). The explanation of different response of sensitive and resistant NBL cells to KDM5B silencing is not clear and will be subject of further studies. However, we hypothesized that it is related to the different expression of this gene in sensitive and resistant NBL cell lines.

There is emerging evidence for the deregulation of KDM5B and the important phenotypic consequences in various types of cancer, making this enzyme a promising factor for the prediction of sensitivity to CDDP. It will be necessary to study the relationship between cisplatin sensitivity and histone methylation to understand resistance to this drug.

Acknowledgements

Not applicable.

Funding

This research was funded by the Grant Agency of Charles University, Czech Republic (grant no. 812217).

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

TE designed and led this study. MB performed RT-qPCR, western blotting, flow cytometry and cell proliferation assays. NP performed siRNA transfection, RT-qPCR, western blotting and flow cytometry. MB and NP analyzed the data and performed the statistical analysis. MB and NP wrote the manuscript. AV designed experiments. MB, NP, AV and TE confirm the authenticity of all the raw data. All authors have reviewed the manuscript and read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.
Competing interests

The authors declare that they have no competing interests.

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