Kdm4c is Recruited to Mitotic Chromosomes and Is Relevant for Chromosomal Stability, Cell Migration and Invasion of Triple Negative Breast Cancer Cells

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ABSTRACT: Members of the jumonji-containing lysine demethylase protein family have been associated with cancer development, although their specific roles in the evolution of tumor cells remain unknown. This work examines the effects of lysine demethylase 4C (KDM4C) knockdown on the behavior of a triple-negative breast cancer cell line. KDM4C expression was knocked-down by siRNA and analyzed by Western blot and immunofluorescence. HCC38 cell proliferation was examined by MTT assay, while breast cancer cells’ migration and invasion were tested in Transwell format by chemotaxis. Immunofluorescence assays showed that KDM4C, which is a key protein for modulating histone demethylation and chromosome stability through the distribution of genetic information, is located at the chromosomes during mitosis. Proliferation assays demonstrated that KDM4C is important for cell survival, while Transwell migration and invasion assays indicated that this protein is relevant for cancer progression. These data indicate that KDM4C is relevant for breast cancer progression and highlight its importance as a potential therapeutic target.

KEYWORDS: Chromosome segregation, jumonji domain-containing histone demethylases, RNA interference, triple-negative breast neoplasms

Introduction

Breast cancer is considered the most common form of malignancy affecting women and is a public health problem accounting for half a million deaths worldwide each year.1 In accordance with the different forms of cancer that are able to systemically affect human beings, the formation of neoplastic cells is associated with changes in gene expression and genomic instability, which are primarily related with epigenetic events that directly alter the structure and function of proteins involved in preserving cell integrity.2,3 Furthermore, a wide number of factors whose expression is altered in tumor lineages when compared with unaffected cells have been identified as potential targets for treating this disease. Among these factors, members of the Jumonji (JMJ) domain-containing protein family have raised special interest due to their relevance for cancer cell phenotypes.4,5 Interestingly, the subset of lysine demethylases (KDM) JMJD2/KDM4 is associated with breast cancer development, as this subset is relevant for cell proliferation as well as for regulating the migratory and invasive abilities of tumor cells.6,7

A member of this important group of macromolecules, the lysine demethylase KDM4C (also known as JMJD2C/GASC1), is an important oncogene that is over-expressed in several forms of human neoplasm including lymphoma, acute myeloid leukemia, colon cancer, lung sarcomatoid carcinoma, and prostate cancer.8–12 KDM4C is the only member of the KDM4/JMJD2 subfamily that is able to interact with mitotic chromosomes, and its over-expression is related to increased chromosomal instability in U2OS cells,13 a common phenomenon in the formation and development of human neoplasms.14,15

In contrast, KDM4C has been directly associated with breast cancer progression through the hypoxia response pathway by co-activating hypoxia-inducible factor-1 (HIF-1)16 and is recognized as a potential prognostic marker for the evolution of the invasive forms of this disease.17,18 Although the specific mechanism involving KDM4C in tumor progression remains to be determined, we have recently demonstrated that inhibiting the activity of KDM4C affects the chromosomal stability and cellular proliferation of triple-negative breast cancer lines.19 Such observations highlight the importance of this protein in the phenotype and survival of breast cancer cells.

In this work, we evaluated KDM4C cellular localization and its effects on cell proliferation, migration and invasion in the HCC38 triple-negative breast cancer cell line, a variant of breast cancer that represents a heterogeneous subtype of malignancy that is generally associated with an aggressive clinical course with limited targeted therapies.20 We found that KDM4C is important for cell proliferation and is able to interact with chromosomes during mitosis, suggesting a role in maintaining correct chromosome segregation. Additionally, migration and invasion assays indicated that KDM4C could be involved in specific invasion pathways.

Methods and Materials

Cell culture and RNA interference

The HCC38 cell line was obtained from American Tissue Culture Collection (ATCC) and grown in Roswell Park Memorial...
Institute (RPMI) 1640-ATCC modification media (Gibco, ThermoFischer Scientific, Waltham, USA) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin at 37°C under a 5% CO₂ atmosphere. After the cells reached 70% confluence, they were transiently transfected with 200 nM siRNA using Lipofectamine® RNAiMAX (Invitrogen, ThermoFischer Scientific, Waltham, USA) according to the manufacturer's protocol. The following two siRNAs targeting KDM4C were able to knockdown its expression: (siRNA1) AGA CCU AGC ACA CUG GAA GCU UCG GAG GAG UUC CGG GAG UUC AAC AAA U and (siRNA2) AGA CCU AGC ACA CUG GAA GCU UCG G. The reduction in KDM4C protein expression was evaluated by western blot. All of the experiments were performed in duplicate, and the results obtained were from at least two independent experiments.

Western blot

HCC38 cells were lysed in a cocktail containing RIPA buffer (1x PBS, 1% Nonidet P-40, and 0.1% SDS) and protease inhibitor complex (Roche, Germany) according to previous reports. After centrifugation, 10 μg of total protein from the supernatant was mixed with an equal volume of sample buffer and denatured at 95°C for 5 min. Then, the samples were separated by 10% SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane with transfer buffer at pH 8.3 (39 mM glycine, 48 mM Tris base, 0.037% SDS, and 20% methanol). After the proteins were transferred, the membrane was incubated in PBS containing 0.1% (v/v) Tween 20 and 5% non-fat dry milk for 1 hour at room temperature. After the membrane was washed, it was incubated with rabbit anti-KDM4C antibody (1:500 dilution; Abcam ab85454) for 2 hour. Next, the samples were washed three times with PBS-T and incubated with HRP-conjugated anti-rabbit IgG (1:2000 dilution; Abcam ab6721) for 1 hour. The membrane was developed using Luminata Crescendo solution (Merck, Massachusetts, USA) and visualized in a MyECL Imager (ThermoFischer Scientific, Waltham, USA) according to the manufacturer's instructions.

Immunofluorescence assays (IFAs)

1x105 HCC38 cells were grown on coverslips and treated with siRNAs as described previously up to 70%-80% confluence. Forty-eight hours post-treatment, the cells were pre-extracted with 0.2% PBS/Tween 100 and fixed in 3.7% formaldehyde for 15 min at room temperature. The fixed cells were permeabilized with 0.5% Triton X-100 in PBS and blocked with 3% bovine serum albumin in PBS for 1 hour. Next, the cells were incubated overnight at 4°C with anti-KDM4C (Abcam ab85454), anti-KDM4A (Abcam 24545) or anti-trimethyl H3K9 (Abcam ab10812) antibodies at 1:250 dilution followed by three washes with PBS containing 0.01% Triton X-100 and then incubation with Alexa Flou 488 (1:500 dilution, Abcam 150077). The cells were washed twice and mounted in ProLong Diamond Antifade Mountant with DAPI (Life Technologies, ThermoFischer Scientific, Waltham, USA). Images were acquired on an Eclipse Ni-E microscope (Nikon, Melville, USA) and analyzed with ImageJ software (NIH) at 40X-100X by triplicate in three independent samples.

Proliferation assays

A previous assay for KDM4A was adapted to analyze breast cancer cell proliferation. HCC38 cells were seeded in 96-well plates and incubated in RPMI 1640 medium (ATCC modification) containing 10% FBS. One day later (day 0), the cells were treated with Lipofectamine RNAiMAX alone or with Lipofectamine RNAiMAX and siRNAs to a final volume of 100μL per well. Simultaneously, a corresponding sample of each treatment was incubated with 20 μL MTT (Life Technologies, ThermoFischer Scientific, Waltham, USA) for an additional 4 hour. Then, the supernatant was removed, and 150μL of dimethyl sulfoxide (DMSO) was added. Absorbance was measured at 550 nm using a microplate reader (Tristar2 LB 942 Multimode Reader, Berthold Technologies, Calsbacher, Germany). After 24 hour and 48 hour, the control and RNAi-treated cells were analyzed with MTT as on day 0.

Migration and invasion assays

The migratory and invasive abilities of HCC38 cells treated with KDM4C siRNAs were evaluated using the following commercially available kits from Millipore (Massachusetts, USA): (1) Chemotaxis Cell Migration Assay Kit (ECM508) and (2) Collagen Cell Invasion Assay Kit (ECM551). Cells were analyzed according to the manufacturer’s instructions 2 hour after transfection with KDM4C siRNAs. Briefly, the cells were trypsinized, re-suspended in serum-free RPMI 1640 medium and seeded on polycarbonate membrane Boyden chamber inserts in a 24-well plate that was either coated with (invasion) or without (migration) collagen (BD Biosciences). Then, 500 μL of RPMI 1640 medium containing 10% FBS was added to the lower chamber to induce chemotaxis, and migrating or invading cells were stained and quantified in a Tistor2 Multimode Reader (LB 942, Berthold Technologies, Calmbacher, Germany) at 550 nm after 48 hour of incubation at 37°C in a 5% CO₂ incubator.

Kaplan–Meier plotting analysis

Survival analysis by Kaplan–Meier was performed with the publicly available database Kaplan–Meier plotter (kmplot.com). This database contains information from a wide number of studies for breast (5143), ovarian (1816), lung (2437) and gastric (1065) cancer patients with a mean follow-up of 69/40/49/33 months. The Kaplan–Meier-Plotter has the capacity to assess the effect of 54,675 genes on patient survival.
by analyzing the data of 10,461 tumor samples. Briefly, KDM4C was tested by using the JetSet probe ‘209984_at’ into the breast cancer–specific area of the database to obtain Kaplan–Meier survival curves, in which the number of patients at risk of mortality was reported below the main plot, in conjunct with the hazard ratios (HR) with 95% confidence intervals (CIs), and P-values (from the log-rank test).

**Statistical analysis**

All results are shown as the means-standard errors, consequential from at least two independent experiments with samples analyzed by triplicate. Statistical comparison was accomplished by analysis of variance (ANOVA) (one-way) and two means evaluation (Student t-test) by using the SPSS 10.0 (IBM), with a significance cutoff of P<.05.

**Results**

*KDM4C is relevant for cell proliferation*

KDM4C relevance in breast cancer cell survival was tested by treating HCC38 cells with siRNAs 1 and 2, followed by evaluation using cell proliferation assays. Twenty-four and forty-eight hours after KDM4C siRNA transfection, a significant reduction in HCC38 cell proliferation (approximately 20% and 40%, respectively) was observed (black bars, Figure 1C) compared with untreated cells (C, Figure 1C), suggesting that KDM4C is associated with HCC38 cell survival. These results agree with previous reports involving other members of the KDM4 subfamily in breast cancer.7,24

*KDM4C associates with mitotic chromosomes and is involved in chromosome segregation*

To evaluate the effect of KDM4C reduction on breast neoplasms, siRNAs against KDM4C were tested in the HCC38 triple-negative breast cancer cell line. Western blot analysis confirmed the expression of KDM4C in the HCC38 cell line control samples, which was significantly reduced by treatment with siRNAs 1 and 2. These observations were also confirmed by IFAs (Figure 1A and B); thus, these siRNAs were used to examine KDM4C-associated HCC38 cell phenotypes.

IFAs showed that KDM4A is excluded from mitotic chromosomes (Figure 1A), while KDM4C interacted with chromosomes during the phases of mitosis (Figures 1A and 2A), highlighting the specific relevance of KDM4C histone demethylase for segregating the genetic information in the triple-negative breast cancer cell line model. In addition, HCC38 cells treated with the KDM4C siRNAs exhibited an increased number of chromosome segregation defects. The number of...
lagging chromosomes (associated with delayed movement during anaphase) increased by 45% and 36% in the siRNA1- and 2-treated cells, respectively, while micronuclei (resulting from mitotic segregation defects) rose to 10%-16% with siRNA treatment. KDM4C knockdown showed a significant increase in H3K9 trimethylated levels, demonstrating a functional role for KDM4C in maintaining this important epigenetic hallmark. In all cases, the chromosomal instability events were significantly higher in KDM4C knockdown cells than in untreated cells (Figure 2B), suggesting that KDM4C activity is relevant for proper segregation of genetic information through mitosis.

**KDM4C knockdown reduces HCC38 cell migration**

HCC38 cell migratory and invasive capacities were tested in the presence or absence of KDM4C by Transwell assays. KDM4C-depleted cells displayed a reduction in migratory ability compared with untreated cells (Figure 3A). However, their invasion through a collagen matrix was increased under the same conditions (Figure 3B). In contrast, HCC38 cells were unable to migrate or invade when serum-free medium was present in the lower Transwell chamber, indicating that no random cell movement was present during the assay.

**Breast cancer patients with high expression levels of KDM4C present a better prognosis**

The importance of KDM4C as a prognostic marker was evaluated by using the database associated with the Kaplan–Meier plotter service. Using the server information, survival curves were plotted for all patients (n = 3951; Figure 4A). Additionally, further curves were plotted, finding significant differences for the following subgroups: estrogen receptor (ER) negative (n = 801; Figure 4B) and lymph node positive (n = 1133; Figure 4C). In these cases, the high expression of KDM4C mRNA was significantly correlated with improved RFS, indicating that lower levels of KDM4C correlate with a higher risk of relapse and the development of a more aggressive form of breast cancer in patients. However, it wasn't observed any difference with statistical relevance for patients with triple-negative breast cancer (n = 255, HR, 0.78; 95% CI, 0.51-1.2; P= .26), maybe due to the low number of data available for this type of patients, as the relevance of KDM4C for this form of breast cancer has been already tested in previous works. With respect to the absence of statistical difference when PR negative (n = 549, HR, 0.76; 95% CI, 0.54-1.05; P= .099) and HER2 negative (n = 800, HR, 0.82; 95% CI, 0.6-1.12; P=.2) were compared, it could suggest an independent pathway for KDM4C role for breast cancer showing these specific phenotypes.
Discussion

The present work showed that KDM4C affects chromosomal segregation and the proliferative, migratory and invasive capacities of a triple-negative breast cancer cell line. The reduction in KDM4C was able to increase histone 3 lysine 9 trimethylation. These data suggest that the expression of genes involved in breast cancer cell mitosis may be modulated by KDM4C demethylase activity. The *Kdm4c* gene, which was described previously as part of the 9p24.3 amplicon, is frequently observed in breast cancer, and KDM4C is widely associated with the development of other cancers including prostate cancer and esophageal cancer.\(^\text{25}\) In concordance, *Kdm4c* gene amplification has also been observed in triple-negative breast cancer,\(^\text{5,26}\) although little is known about its specific role in the development of this cancer. This study is the first to describe the effect of KDM4C knockdown on a triple-negative breast cancer cell line (HCC38), namely, an increase in chromosome segregation defects that affect HCC38 cell survival. These observations demonstrate the high relevance of KDM4C for maintaining the phenotype of one of the most aggressive forms of breast neoplasm.

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**Figure 3.** Migration and invasion assays. (A) Migration assays of KDM4C-depleted or untreated cells, performed according to the manufacturer’s recommendations. Representative stained migrating cells are shown in each sample with the quantification at OD 550, with a clear reduction in HCC38 migration ability after siRNAs treatment. C: Negative control, C+: Positive control, siRNA1, siRNA2: siRNAs treatments (B) Invasion assays for HCC38 cells in the presence or absence of KDM4C. Illustrative images are presented with their corresponding quantification. Data shown represent the means ± standard errors of the means of data from at least 3 independent experiments. *P < .05; **P < .005. These observations confirm an increment of HCC38 invasiveness after KDM4C depletion.

**Figure 4.** Kaplan-Meier plotting. Survival graphs of breast cancer based on KDM4C expression for all breast cancer cases (A) and in function of estrogen receptor negativity (B) or lymph nodes invasion (C). Using publicly available database (KM plotter; www.kmplot.com), relapse-free survival curves were analyzed by KDM4C expression. HR and Log-rank *P*-values were calculated in kmplot database and are presented for each graph.
Recently, Kupershmit et al. demonstrated that KDM4C is the only member of KDM4 histone demethylase subfamily that is able to interact with chromosomes during mitosis in osteosarcoma cells and that its depletion or over-expression leads to an increase in chromosome segregation errors. Similarly, KDM4C is actively recruited to mitotic chromosomes and remains associated to them through with the different phases of chromosome segregation (Figures 1A and 2A). In contrast, KDM4A is completely excluded from mitotic chromosomes when this protein is analyzed under similar conditions than KDM4C (Figure 1A). Such observations strongly indicate that KDM4C is the only member of KDM4 subfamily which actively participates in mitosis process. Intending to characterize this protein’s localization and function during mitosis in breast cancer, we transiently depleted KDM4C and then evaluated the different mitotic errors by IFAs. KDM4C translocates to the nucleus during interphase (Figure 1B) and interacts with the chromosomes along the evolution of cellular division during mitosis (Figure 2A), indicating that KDM4C could play an important role in distributing genetic information in breast cancer cells.

These observations were supported by an increase in the number of chromosome segregation-associated errors, primarily an increased number of lagging chromosomes and the presence of micronuclei (Figure 2B). These results strongly suggest that aneuploidy and chromosome instability, which are commonly observed in breast cancer cells, could be associated with the changes in KDM4C activity during mitosis. Similarly, we also observed a significant increase in the trimethylated form of H3K9 in siRNA-treated HCC38 cells (Figure 2C), which has also been reported under similar conditions in other neoplastic cell lines. However, additional studies are required to identify the specific role of this demethylase for the proper segregation of DNA content in daughter cells, as over-expressing KDM4C has also been shown to affect mitosis in the U2OS osteosarcoma cell line.

In contrast, KDM4C depletion has been associated with a reduction in the proliferation of different types of cancer, including breast cancer cell lines positive for her2/neu. We demonstrated that KDM4C depletion is also relevant in a breast cancer cell line negative for her2/neu, progesterone and ERs (Figure 1C), suggesting that KDM4C could be associated with breast cancer proliferation in a her2/neu-independent pathway that remains to be determined, as KDM4C is involved in the regulation of specific genes that mediate cell growth.

Because KDM4C is important for cell proliferation and chromosome segregation, we also evaluated its potential role in HCC38 migration and invasion. The active and complex invasion process involves the polarization and formation of cell protrusions (also termed invadopodia) that recognize and interact with the extracellular matrix (ECM) and is mediated by different integrins, followed by the recruitment of a wide number of macromolecules that are able to degrade the ECM, thus facilitating invasive cell movement.

Different studies performed with another member of the KDM4 subfamily, KDM4A, have demonstrated its relevant role in migration and invasion assays, as its depletion resulted in a reduction in both events in breast cancer. Remarkably, under similar experimental conditions, KDM4C depletion also reduced the number of migrating cells in Transwell assays (Figure 3A). However, similar results were not observed in HCC38 cancer cell invasion (Figure 3B). It is known that aggressiveness of tumoral cells is directly associated with their invasion ability, as a logical step in the metastasis of localized tumors and colonization of different organs in the terminal phases of the disease, which could be associated with the variable expression levels of this protein. Remarkably, the expression profile of KDM4C mRNA expression was studied by Kaplan–Meier plotting. Interestingly, samples of breast cancer showing low levels of this mRNA are significantly associated with tumor recurrence in patients with breast cancer, and for specific cases in conjunct with their TP53 mutation status, ER expression status and lymph node invasiveness. These results indicate that KDM4C inhibits the progression of breast cancer, controlling their aggressiveness as an independent function of the different phenotypes that can be observed for the different forms of breast cancer. The association of high levels of KDM4C with a better prognosis in breast cancer patients was also observed by Berdel et al. who elegantly demonstrated by immunohistochemistry studies performed with this protein in 355 breast cancer samples presenting different histological types and grades, ER, progesterone receptor (PR) and HER2 status, that tumors with KDM4C negativity present a better prognosis and are associated to less aggressive tumors than KDM4C-positive samples, as well as KDM4C mRNA levels are higher in lower histological grades of breast cancer. These observations suggested that KDM4C could be an important prognostic marker for the aggressiveness of breast cancer tumor subtypes, which is in agreement with the reduction in the invasive capacity of HCC38 cancer cells in presence of KDM4C and highlights the relevance of further analysis of KDM4C’s role for breast cancer cells evolution.

Such behavior suggests that KDM4C could be associated with the expression of the proteins that participate in the pathways regulating the cellular migration/invasion. Belonging to the different pathways associated with these important processes, the Src kinase-FAK (Focal Adhesion kinase) route has raised a special interest. Interestingly, it has been demonstrated that downregulating FAK in melanoma cells produce a similar phenotype to the one observed for KDM4C: a promotion of the invasiveness and a reduction of cellular migration, being a one potential association between these important macromolecules involves the inositol hexakisphosphate kinase 1 (IP6K1), whose deletion affects carcinoma migration and invasion by reducing FAK activation, while the expression of IP6K1 is associated with the dissociation of KDM4C from chromatin increasing H3K9me3 levels.
On the other hand, KDM4C is also an important epigenetic regulator, involved in the maintenance of human ESCC-initiating cells by enhancing SOX2 expression among other targets. Remarkably, SOX2 has been associated with the suppression of the invasiveness of breast cancer cells in function of its transcriptional activity, which could represent an additional route for KDM4C activity during breast cancer cells migration and invasion. The test of these interesting pathways goes further of the scope of this work, but they establish a promising starting point for new research routes in the analysis of KDM4C as anticancer drugs target.

In summary, we have determined the effects of KDM4C depletion in cellular division, proliferation and migration in a tumor-node-metastasis (TNM) stage IIB, grade 3, primary ductal triple-negative carcinoma cell line, highlighting the important role of this histone demethylase in breast cancer development and survival. Altogether, these results highlight the potential of KDM4C as a promising therapeutic target against the most aggressive forms of breast neoplasm.

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Author Contributions
JG contributed to study design, conducting experiments, interpretation of the results and manuscript writing; FL contributed to concept development, experimental design, manuscript writing and funding for the project.

REFERENCES
1. WHO. World Cancer Report. Geneva, Switzerland: World Health Organization; 2008.
2. Lizzano F, Garcia J. Epigenetic control and cancer: the potential of histone demethylases as therapeutic targets. Pharmaceuticals. 2012;5:963–990.
3. Zaidi SK, Van Wijnen AJ, Lian JB, Stein JL, Stein GS. Targeting deregulated transcriptional repressor KDM4C as a therapeutic strategy for aggressive soft tissue sarcomas. J Cell Physiol. 2008;214:134–141.
4. Liepka EC, Misteli T. KDM4C promotes metastasis in a breast cancer cell line through the histone demethylase JMJD2B. Cancer Res. 2015;75:206–216.
5. Berry WL, Janknecht R. KDM4/JMJD2 histone demethylases: epigenetic regulators in cancer cells. Cancer Res. 2013;73:2936–2942.
6. Li BX, Zhang MC, Luo CL, et al. Effects of RNA interference-mediated gene silencing of JMJD2A on human breast cancer cell line MDA-MB-231 in vitro. J Exp Clin Cancer Res. 2011;30:90.
34. Jadav RS, Kumar D, Buwa N, et al. Deletion of inositol hexakisphosphate kinase 1 (IP6K1) reduces cell migration and invasion, conferring protection from aerodigestive tract carcinoma in mice. *Cell Signal*. 2016;28:1124–1136.

35. Burton A, Azevedo C, Andreassi C, Riccio A, Saiardi A. Inositol pyrophosphates regulate JMJD2C-dependent histone demethylation. *Proc Nat Acad Sci U S A*. 2013;110:18970–18975.

36. Yuan X, Kong J, Ma Z, et al. KDM4C, a H3K9me3 histone demethylase, is involved in the maintenance of human ESCC-initiating cells by epigenetically enhancing SOX2 expression. *Neoplasia*. 2016;18:594–609.

37. Wu F, Ye X, Wang P, et al. Sox2 suppresses the invasiveness of breast cancer cells via a mechanism that is dependent on Twist1 and the status of Sox2 transcription activity. *BMC Cancer*. 2013;13:317.