SMC1A promotes tumor growth in breast cancer by activation of the AKT/FOXN1/STN1 Signaling Pathway

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Abstract

Background

SMC1A (Structural maintenance of chromosomes 1) is overexpressed in various cancers and acts as an oncogene which has been implicated in critical biological functions (cell-cycle checkpoints regulation, cell division, and DNA repair). However, the mechanism and role of SMC1A in breast cancer are poorly understood.

Methods

TCGA database was utilized to explore the expression of SMC1A and the relationship between SMC1A and FOXM1 and STMN1. Subsequently, short hairpin RNA (shRNA) targeting SMC1A was used to examine the biological functions of it in MDA-MB-231 and MDA-MB-468 cells. Finally, subcutaneous xenograft model to verify the roles of SMC1A *in vivo*.

Results

In the present study, we demonstrated that SMC1A was significantly increased in breast cancer (BC) via TCGA database. Then loss and gain of function studies revealed that SMC1A contributed to BC cell survival, apoptosis, and invasion. Interestingly, we found that SMC1A triggered the AKT/FOXM1 cascade, which promoted BC cell proliferation. Furthermore, overexpression of FOXM1 abolished the inhibition of cell growth induced by SMC1A silencing *in vitro*. Clinically, the expression of SMC1A in BC tumor tissues is positively correlated with the expression of FOXM1.

Conclusion

Taken together, our findings not only enhanced our understanding of molecular mechanisms of SMC1A in BC, but also might provide a novel target for the development of therapeutic strategies.

Background

Breast cancer (BC) is the most diagnosed cancers in women worldwide, accounting for > 2 million new cancer cases and with increase of the incidence of advanced breast cancer (ABC) in women aged 25 to 39 years [1, 2]. The 5-year survival rates of BC have proven effective to improve with the introduction of adjuvant and neoadjuvant therapies, but most of the patients with advanced or metastatic stage which is largely incurable [3–6]. The poor outcome for BC patients is due to the high recurrence or develop metastases and drug resistance. Herein, it is imperative to explore potential mechanism of BC tumorigenesis and develop novel therapeutic strategies for this cancer.

The structural maintenance of chromosomes protein 1A (SMC1A), belongs to the SMC family members, is linked to the proper cohesion, and involved in the structural maintenance of chromosome during cell division [7, 8]. It has been found that SMC1A was a conserved member of the cohesion multiprotein complex, which has important roles in maintaining genome stability by regulating chromosome function, gene regulation and double-stranded DNA repair [7, 9, 10]. Accumulating evidence has identified that aberrant expression and mutation of SMC1A was associated with a series of human diseases, including Cornelia de Lange syndrome and malignant carcinomas [11–13]. With more deep understanding of roles and molecular mechanisms of SMC1A in cancers, SMC1A was frequently upregulated in multiple cancers, and is emerged as an important molecule implicated in the initiation and progression of cancer [14–16]. Previous study has shown that SMC1A could bind with BRCA1 to regulate DNA-damage response and cell cycle checkpoint-mediated DNA repair in response to x-irradiation and chemotherapeutic agents [17]. Though SMC1A was found to upregulate and knockdown of SMC1A inhibited cell migration, epithelial-mesenchymal transition (EMT) and drug sensitivity in BC, the important roles and detailed mechanisms of SMC1A in the tumorigenesis and progression of BC remains poorly understood.

In the present study, we investigated the expression and outcome of SMC1A based on public dataset analysis. Further experiments were performed to explore the biological functions of SMC1A via SMC1A silencing *in vitro* and *in vivo*. Importantly, we demonstrated that SMC1A exerted an oncogene by promoting transcription factor Forkhead box M1 (FOXM1), which plays important roles in tumor progress. Our results elucidated a novel SMC1A/FOXM1 regulatory mechanism and might shed light on the avenue to target oncogenic pathways in cancers.

Materials And Methods

Bioinformatics analysis

The differential gene expression in tumor and normal tissues and the correlation between SMC1A, FOXM1 of patients with BC were analyzed by GEPIA (<http://gepia.cancer-pku.cn/>) tool [18]. The protein expression of SMC1A was obtained from CPTAC (Clinical Proteomic Tumor Analysis Consortium) and The Human Protein Atlas (<https://www.proteinatlas.org/>). The genes related SMC1A from LinkedOmics (<http://www.linkedomics.org>) [19]. Gene set enrichment analysis (GSEA) tool was used to conduct KEGG pathways. Kaplan-Meier Plotter (<http://kmplot.com/analysis/index.php>) was used to assess the prognosis of patients with BC.

Cell lines and cultures

Two human BC cell lines (MDA-MB-231 and MDA-MB-468) were purchased from the Chinese Academy of Sciences Shanghai Institute of Life Sciences. All cells were cultured at 37°C in a humidified atmosphere of 5% CO₂ in the appropriate medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S) solution.

Plasmid constructions and Lentiviral Production

The lentiviral vector (pLKO.1-TRC Cloning Vector) carrying shRNA targeting human SMC1A was obtained from Sigma Aldrich (shRNA#1, sense, 5'-CGACAGATTATCGGACCATT-3', shRNA#2, sense, 5'-GCCGGGACTGTATTCAGTATA-3'). The cDNA of FOXM1 was cloned to pCDNA3.1 vector and pCDNA3.1 vector was used as a control. All plasmids were identified by sequencing. Lentivirus was produced in HEK-293T cells and harvested at 48 h after transfection. Lentiviral particles with shRNAs or Negative Control (SCR) were added into MDA-MB-231 and MDA-MB-468 cells, the medium with lentivirus particles was replaced with refresh growth medium after 24 h of infection. Stable cell lines were selected out in 2 µg/mL puromycin (Sigma Aldrich, St. Louis, MO, USA) for 4 days.

Cell proliferation assay

Cell Counting Kit-8 (CCK-8) (Dojindo, Japan) assay was used to evaluate cell proliferation ability. MDA-MB-231 and MDA-MB-468 cells with or without SMC1A silencing were seeded into 96-well plates with the number of 2×10^3 . Subsequently, 90 µl basic medium with 10 µl CCK-8 replaced the medium. After incubation at 37 °C for 2 h, the absorbance at 450 nm was measured with a microplate reader (BioTek Instruments, USA).

Colony formation assay

MDA-MB-231 and MDA-MB-468 cells with or without SMC1A silencing were plated into each well of a 6-well culture plate (600 cells/well), with the medium refreshed every 3 days. Cells were fixed with 4% paraformaldehyde for 15 min and visualized by 0.5% (w/v) crystal violet (Sigma-Aldrich) staining when clones were visible. The number of colonies was quantified by Image J software.

Flow cytometry

For cell apoptosis, the apoptotic rate of tumor cells was stained using Annexin V Apoptosis Detection Kit II (BD Biosciences) according to the manufacturer's protocol. Finally, the apoptotic cells were analyzed by flow cytometer (BD Biosciences, CA, USA).

For cell cycle analysis, MDA-MB-231 and MDA-MB-468 cells with or without SMC1A silencing were stained by PI-staining solution after fixing in 75% ethanol at 4 °C overnight, and then the percentages of cells in the G0-G1, S, and G2-M phase were counted using a flow cytometer.

In vivo experiments

All animal studies were approved by the Institutional Animal Care and Use Committee (IACUC) of Tongji University and conducted under the National Institutes of Health. BALB/c nude mice aged 4 weeks were purchased from the Shanghai SLAC Laboratory Animal Co., Ltd (Shanghai, China). Approximately 1×10^7 MDA-MB-231 cells transduced with SCR or SMC1A shRNA#1 were subcutaneously injected into the right flank of nude mice. Tumor size was examined every week. The volume was calculated using the formula

$V = \text{length} \times \text{width}^2 / 2$. All mice were sacrificed 4 weeks later, and the xenografts were dissected and weighed.

Western Blotting Analysis

Western blotting was carried out as previously described [20]. In brief, a total of 50 µg of protein was isolated by 10% SDS-PAGE and then transferred into PVDF membranes (Millipore, Billerica, MA, USA). The membranes were incubated with rabbit anti-SMC1A (ab21583, Abcam), rabbit anti-STMN1 (11157-1-AP, Proteintech), rabbit anti-FOXO1 (13147-1-AP, Proteintech), rabbit anti-AKT (#491, Cell Signaling Technology) or rabbit anti-Phospho-Akt (Ser473) (#4060, Cell Signaling Technology) at 4 °C overnight after blocking. The signals were visualized using ECL chemiluminescent reagents (Thermo Fisher Scientific, Inc.) by ImageQuant™ LAS 4000 mini (GE Healthcare).

Immunohistochemical (IHC) analysis

Immunohistochemistry was performed as our previous described [21].

Statistical analysis

The data was expressed as mean ± standard deviation (SD). All experiments were repeated at least three independent experiments. All statistical analyses were performed using GraphPad Prism software (version 5; GraphPad Software Inc., La Jolla, CA, USA). Student's T-test was used to compare two groups, and one-way ANOVA was used to compare three or more groups. A p-value < 0.05 was considered to indicate significance.

Results

SMC1A expression is upregulated and associated with unfavorable prognosis in BC

To investigate the significance of SMC1A in breast cancer, we evaluate SMC1A mRNA expression in TCGA database at GEPIA web (<http://gepia2.cancer-pku.cn/#inde>). As shown in Figure1A, the mRNA level of SMC1A was significantly upregulated in most of cancers, including breast cancer. Subsequently, the protein expression of SMC1A between BC tissues and adjacent tissues was compared through the public tumor database CPTAC Data Portal (<https://cptac-data-portal.georgetown.edu/>). The data also showed that the expression of SMC1A was increased in BC tissues in comparison with matched adjacent tissues (Figure1B, left). The immunohistochemical staining of SMC1A in BC tissues was shown obtained from The Human Protein Atlas (<https://www.proteinatlas.org/>) (Figure1B, right). Furthermore, the Kaplan-Meier analysis showed that patients with high SMC1A abundance had obviously shorter recurrence-free survival (RFS) and distal metastasis-free survival (DMFS) than patients with low high SMC1A abundance from TCGA database at Kaplan-Meier Plotter (<http://kmplot.com>) (Figure 1C, D). Taken together, these results strongly suggested that SMC1A might play a crucial role in the progression of BC.

SMC1A promotes proliferation of breast cancer cells *in vitro*

Considering that clinical results and our previous study supported a potential pro-tumorigenic role of SMC1A in BC. To address this, MDA-MB-231 and MDA-MB-468 which had higher endogenous SMC1A were chosen for knockdown experiments. As shown in Figure 2A, two independent shRNAs targeting SMC1A (shSMC1A#1, shSMC1A#2) were introduced into MDA-MB-231 and MDA-MB-468 cells and the protein expression of SMC1A was significantly reduced after shRNAs transduction, which confirmed the effectiveness of our loss-of-function assay. Decreased expression of SMC1A significantly inhibited the proliferation of BC cells (Figure 2B). Furthermore, colony formation assays indicated that SMC1A silencing significantly reduced the number and the size of cell clones (Figure 2C). Emerging evidence have shown that alteration of apoptosis and cell-cycle distribution were two key factors that could contribute to the tumorigenesis of cancer cells. Herein, flow cytometric assays were performed to examine the roles of SMC1A in BC cell apoptosis and cell-cycle distribution. Notably, Annexin V-PI Flow cytometric experiment showed depletion of SMC1A resulted in an obviously increased in apoptosis rate (Figure 2D). Apart from cell apoptosis, SMC1A silencing had a higher percentage of cells in the G2 phase in both MDA-MB-231 and MDA-MB-468 cells, which indicated the induction of cell arrest at the G2 phase after SMC1A knockdown (Figure 2E). These data suggested that SMC1A promoted tumor cell proliferation by regulating cell apoptosis and cell cycle of BC cells *in vitro*.

Knockdown of SMC1A suppresses tumorigenesis *in vivo*

To examine the oncogenic roles of SMC1A *in vivo*, MDA-MB-231 cells were selected to establish the xenograft model in nude mice. Cells infected with pLKO.1-control (SCR) or shSMC1A#1 lentivirus were subcutaneously injected into the BALB/c nude mice. Xenograft tumors were shown at 4 weeks after inoculation (Figure 3A). It was shown that the tumor growth curve and weights significantly decreased after SMC1A knockdown (Figure 3B and C), indicating that the SMC1A was required for tumor growth *in vivo*. Moreover, IHC analysis of xenograft tissue sections showed a much lower SMC1A expression in xenograft tissues with the depletion of SMC1A. These findings demonstrated that knockdown of SMC1A suppressed the growth of BC *in vivo*, consistent with the *in vitro* results.

SMC1A knockdown suppresses FOXM1 expression

To explore the mechanism underlying the effects of SMC1A on the proliferation of BC cells, the downstream functional proteins need further investigation. By using RNA-seq data from TCGA database at LinkedOmics web (<http://www.linkedomics.org/>), heat maps were constructed by the top 50 positively and negatively genes associated with SMC1A (Figure 4A). According to the Gene set enrichment analysis (GSEA)-based KEGG signaling pathway enrichment analysis, SMC1A was closely related to cell cycle transition, Ubiquitin mediated proteolysis, miRNAs in cancer and Oocyte Meiosis (Figure 4B and C). Previous studies have shown that FOXM1 was significantly upregulated and played important roles in the process of cell cycle regulation. We wondered to know whether there is a functional link between SMC1A and FOXM1 in BC. The results showed that, following SMC1A knockdown, the expression of FOXM1 was significantly reduced, accompanied with the decreased expression of p-AKT and FOXM1 (Figure 4D). TCGA database analysis showed that transcription levels of FOXM1 were remarkably higher in BC tissues

compared to adjacent normal tissues (Figure 4E). Furthermore, Pearson correlation analysis showed that a strong positive correlation was observed between SMC1A and FOXM1 expression in published human BC gene expression datasets (Figure 4F). Kaplan-Meier analysis showed that patients with high FOXM1 expression had obviously shorter recurrence-free survival (RFS) and distal metastasis-free survival (DMFS) than patients with low high FOXM1 expression based on Kaplan-Meier Plotter (<http://kmplot.com>) (Figure 4G and F). Taken together, these data indicated that SMC1A affected the expression of oncogenic FOXM1 in BC, which involved in tumor progression.

FOXM1 overexpression partially restores the proliferative ability of BC cells following SMC1A knockdown

To confirm that SMC1A functions via the FOXM1, FOXM1 was transfected into SMC1A stably silenced MDA-MB-231 and MDA-MB-468 cells. Western blot was performed to assess the expression of SMC1A, FOXM1, p-AKT and STMN1. The results showed that enforced FOXM1 expression could attenuated the effects of SMC1A on p-AKT and STMN1, characterized by upregulation of p-AKT and STMN1 (Figure 5A). However, there was no obvious change in the expression of t-AKT and SMC1A (Figure 5A). CCK-8 and colony formation assay were performed to test cell proliferative ability and our data showed that the cell viability decreases with SMC1A depletion, and then raises up with FOXM1 overexpression (Figure 5B). Colony formation assay also showed the similar results (Figure 5C). On the other hand, overexpression of FOXM1 partially rescued the apoptotic levels in SMC1A stably silenced MDA-MB-231 and MDA-MB-468 cells (Figures 5D). It was showed that overexpression of FOXM1 in SMC1A-knockdown cells partially rescues the cell cycle arrest (Figures 5E), indicating that FOXM1 was involved in SMC1A regulated cell growth. These data indicated that SMC1A positively regulated FOXM1 to mediate BC progress.

Discussion

Our previous study has shown that increased expression of SMC1A was observed in triple-negative breast cancer (TNBC), gain-of-function of SMC1A could promote the progress of BC by inducing epithelial-mesenchymal transition [20]. In our present study, the expression and clinical features of SMC1A were investigated by TCGA public database. Then the results of lose-of-function of SMC1A inhibited cell growth of BC cells *in vitro* and *in vivo*. Further investigation showed that SMC1A silencing also promoted cell apoptosis and suppressed cell cycle progression at G2 phase in BC cells. In addition, SMC1A contributed to the increase expression of FOXM1 and rescue of FOXM1 could attenuate the phenomenon induced by SMC1A silencing. These findings indicated that SMC1A exert oncogenic activity partly by regulating FOXM1 expression in BC.

A growing body of evidence suggested that SMC1A was taken part in various cellular functions including growth, migration, apoptosis, and phenotypic transition, which is an important step of tumor progress [14, 15, 17, 20]. Down-regulation of SMC1A expression inhibited growth of lung cancer cells via G1/S cell cycle phase arrest and apoptosis pathways [22]. Cohesion complex has been reported to regulate DNA damage-induced intra-S phase and G2/M checkpoints in mammalian cells because it facilitates the recruitment of SMC1A involved in cell cycle checkpoints [23–25]. Consisted with these results, our data

showed that the deletion of SMC1 restrained cell growth by mediating cell apoptosis and cell cycle of BC cells. The xenograft nude mouse models were established by subcutaneous injecting also confirmed the efficacy of the anti-tumor growth of SMC1A silencing *in vivo*.

Next, we further investigated the potential molecular mechanisms of SMC1A involved in alteration of cell apoptosis and cell cycle. Accumulating evidences showed that the rapid development of modern bioinformatics facilitated the large-scale and comprehensive analysis of data, which might provide great convenience to research [18, 19, 26]. We verified that FOXM1 acted as a downstream molecular of SMC1A because FOXM1 was transcription factor primarily that was upregulated in BC and regulated the proliferation-associated gene cluster critical to G2/M transition, mitotic spindle assembly, and chromosome segregation [27, 28]. FOXM1 was reported to involve in a variety of biological processes, including cell cycle progression, apoptosis, angiogenesis, metastasis, and drug resistance [29]. Furthermore, FOXM1 was widely expressed in actively proliferating tissues with potential oncogenic property [30, 31]. In our results, inhibition of SMC1A resulted in a decrease of FOXM1, as well as downstream STMN1. Interestingly, the restore of FOXM1 in SMC1A silencing BC could effectively recover the biological behavior induced by knockdown of SMC1A. Taken together, our results suggested that SMC1A promoted the growth of BC cells via upregulation of activation of FOXM1 expression.

Conclusion

In summary, we identified SMC1A was upregulated and correlated with poor prognosis in patients with BC by public database. Further investigation showed that SMC1A knockdown inhibited the proliferative ability of BC cells by regulating cell apoptosis and cell cycle. In addition, our data indicated that FOXM1 played crucial roles in SMC1A regulated BC cell growth and carcinogenesis.

Declarations

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Contributions

Kaichun Li and Bingxiang Wu supervised the project and contributed to the design and interpretation of all experiments. Qingliang Fang and Long Liu contributed to the design, conduct, and interpretation of all experiments. Ping Dai, Lin Xue, and Shiyu Cheng contributed to experimental studies. Ping Dai, Long Liu and Shiyu Cheng contributed to data analysis. All authors read and approved the final manuscript.

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Ethics declarations

Ethics approval and consent to participate

This study was approved by Shanghai Fourth People's Hospital, School of Medicine, Tongji University, Shanghai, China.

Consent for publication

All authors read and approved the final manuscript.

Competing interests

The authors declared no financial conflict of interest.

Supplementary Information

None.

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Figures

Figure 1

SMC1A was upregulated and associated with poor prognosis. (A) The increased mRNA level of SMC1A in most of cancers was obtained using the GEPIA database (<http://gepia2.cancer-pku.cn/#inde>). (B) The protein expression of SMC1A was analyzed by CPTAC (Clinical Proteomic Tumor Analysis Consortium) and the IHC staining of SMC1A derived from The Human Protein Atlas (<https://www.proteinatlas.org/>).

Kaplan-Meier curve for the RFS (C) and DMFS (D) of patients with BC from Kaplan-Meier plotter databases.

Figure 2

Knockdown of SMC1A inhibited cell proliferation and triggered apoptosis in BC cells in vitro. (A) SMC1A expression in BC cells transduced with SMC1A shRNA was detected by western blot. GAPDH was used as a loading control. (B) Cell proliferation and (C) Colony-formation assays were performed to determine the cell proliferation of BC cells when endogenous SMC1A was silenced. Flow cytometer was performed to determine the cell apoptotic rate (D) and cell cycle distribution (E) of BC cells when endogenous SMC1A was silenced. *p < 0.05, **p < 0.01.

Figure 3

SMC1A knockdown inhibited BC xenograft growth. (A) Images of xenograft tumors from mice in the SMC1A knockdown in vivo. Growth curves (B) and weights (C) of xenograft tumors were shown. (D) Examples of xenograft tumors were stained with SMC1A by IHC. ** $p < 0.01$.

Figure 4

SMC1A knockdown suppressed FOXM1 expression. (A) The heat map showed the first 50 pathways positively (left) and negatively (right) related to SMC1A by online tool (<http://www.linkedomics.org/admin.php>). (B) Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis of SMC1A showed SMC1A associated with cell cycle, miRNA in cancer, ribosome, and oxidative Phosphorylation. (C) Gene set enrichment analysis (GSEA) showed the relationship between SMC1A and

cell cycle. (D) Western blot analysis of p-AKT, total AKT, FOXM1, and STMN1 was determined after SMC1A silencing. (E) Elevated expression levels of FOXM1 were observed by GEPIA (<http://gepia2.cancer-pku.cn/#inde>). (F) The relationship between SMC1A and FOXM1 was analyzed by GEPIA. Kaplan-Meier curve for the RFS (G) and DMFS (H) of patients with BC from Kaplan-Meier plotter databases.

Figure 5

Overexpression of FOXM1 partially restored the biological functions of BC cells following SMC1A knockdown. BC cells were infected with lentiviruses carrying shRNA against SMC1A, then transfected with or without FOXM1. (A) The expression SMC1A, p-AKT, total AKT, FOXM1, and STMN1 was

determined by Western blot. CCK-8 (B) and Clone formation assays (C) were performed to investigate the role of the FOXM1 in the cell proliferation of SMC1A silenced BC cells transfected with or without FOXM1. Flow cytometer were performed to determine the cell apoptotic rate (D) and cell cycle distribution (E) of SMC1A silenced BC cells transfected with or without FOXM1. * $p < 0.05$, ** $p < 0.01$.