Mps1 contributes to tamoxifen resistance in Breast Cancer through phosphorylation of ERα

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Abstract

Overexpression of mitotic kinase monopolar spindle 1 (Mps1) has been identified in many tumor types and targeting Mps1 for tumor therapy has been shown great promise in multiple preclinical cancer models. However, the role of Mps1 in tamoxifen resistance in breast cancer has never been reported. Here in this study, we report that Mps1 determined the sensitivity of breast cancer cells to tamoxifen treatment. Mps1 overexpression rendered breast cancer cells more resistant to tamoxifen, while Mps1 inhibitor or siMps1 oligos could overcome tamoxifen resistance. Mechanistically, Mps1 interacted with ERα and stimulated its transcriptional activity in kinase activity-dependent manner. Mps1 was responsible for ERα phosphorylation at S559 and T224 sites. Importantly, Mps1 failed to enhance the transcriptional activity of ERα in the presence of ERα S559A or T224A mutant. Collectively, our findings suggest that Mps1 contributes to tamoxifen resistance in breast cancer and is a potential therapeutic to overcome tamoxifen resistance in breast cancer.

Introduction

Mps1 is a mitotic serine/threonine kinase acting at an early step in the spindle assembly checkpoint (SAC) and influences centrosome assembly in mammals. The kinase activity of Mps1 is required for both SAC generation and maintenance[1]. Mps1 also plays multiple roles in centrosome duplication, DNA damage, cytokinesis and SMAD signaling response[2-5]. Previous study indicated that the mRNA level of Mps1 are elevated in gastric cancer, papillary thyroid cancer, lung cancer and breast cancer[6-8]. In particular, high levels of Mps1 correlate with a with higher tumor grade in breast cancer[9]. Reducing Mps1 level induces massive apoptosis, while overexpression of Mps1 enable tumor cells to escape apoptosis through its roles in SAC[10]. Thus, elevated Mps1 levels correlate with increased cell proliferation and tumor aggressiveness[11]. However, the role of Mps1 in ERα signalling and endocrine therapy resistance of breast cancer remains unknown.

ERα is a nuclear transcription receptor, which is expressed in about 70% of breast tumors[12]. When estrogen binding to ERα, ERα enter the nucleus to allow transcriptional activation of ERα target genes containing estrogen-responsive elements (EREs) on the promoters in a dimeric form[13]. The phosphorylation of ERα has been shown to influence the ability to bind EREs and gene expression[14]. Current endocrine therapies for ERα positive breast cancer are mainly based on targeting the ERα signalling pathway[15]. The selective estrogen receptor modulator tamoxifen was the most commonly used endocrine therapy drugs[16]. Tamoxifen inhibits breast cancer growth by competing with estradiol (E2) for binding of ERα[17]. However, a substantial proportion of ERα positive breast cancers become resistant to anti-estrogens and tamoxifen resistance presents a major challenge in the treatment of ER-positive breast cancers[18].

In this study, we demonstrated a critical role of Mps1 in the regulation of tamoxifen sensitivity of breast cancer cells. Mps1 interacted with ERα and phosphorylated ERα mainly at Ser559/Thr224 sites. The
phosphorylation of ERα by Mps1 resulted in the stimulation of ERα transactivation activity in the absence of estrogen. These data indicate that Mps1 plays important role in tamoxifen resistance by regulation of ERα transcriptional activity and provides insights into potential adjuvant therapeutic target in breast cancer endocrine therapy.

Results

**Mps1 determines the sensitivity of breast cancer cells to tamoxifen**

The high levels of Mps1 in breast cancer prompted us to assess the role of Mps1 in tamoxifen resistance. To this end, the effect of Mps1 on the growth ability of breast cancer cells to form colonies was first analyzed. As we can see, the number of colonies formed was decreased sharply in cells transfected with Mps1 siRNA oligos (siMps1) compared with cells transfected with siRNA negative control. Notably, Mps1 ectopic introduction led to a substantial increased clonogenicity (Figure 1A). In wounding healing assay, siMps1 MCF-7 cells greatly inhibited migratory capacity to the wounded areas, while the migratory capacity to the wounded areas was greatly increased by Mps1 overexpression (Figure 1B-C), indicative a positive role of Mps1 on cell migration. To further assess the role of Mps1 in the resistance of tamoxifen-induced growth inhibition and cell death. MCF-7 cells were transfected with Mps1 expression plasmid or siMps1 RNA oligos in the presence of tamoxifen and growth ability was measured under different conditions. Colony formation assay showed that Mps1 overexpression apparently made the cells more resistant to tamoxifen-induced cell death, while Mps1 knockdown cells remained hypersensitive (Figure 1A). Consistently, whereas tamoxifen treatment inhibited the migratory capacity of control cells, this effect was significantly enhanced in siMps1 cells and was significantly inhibited by Mps1 overexpression (Figure 1B-C).

Since inhibition of Mps1 by siRNA increased the sensitivity of breast cancer cells to tamoxifen treatment, we next want to investigate whether a specific Mps1 kinase inhibitor Reversine could overcome tamoxifen resistance and show a potential synergistic effect with tamoxifen. To do so, MCF7 cells were treated with various concentrations of reversine in the presence or absence of tamoxifen. Cell survival assay showed that reversine caused a dose-dependent inhibition of cell survival with IC50 values of 0.13 mmol/l, with tamoxifen at IC50 0.25 mmol/l. However, combination treatment of cells with reversine and tamoxifen resulted in significant cell death with IC50 values of tamoxifen at 0.02 mmol/l (Figure 1D). Focus formation analysis also showed that a combined inhibition of tamoxifen and reversine achieved considerable synergy effect in MCF7 and ZR751 cells (Figure 1E). Thus, the results indicated that the level of Mps1 determines the sensitivity of breast cancer cells to tamoxifen.

**Mps1 stimulates the transcriptional activity of ERα**

Regulation of ERα signaling is one of the major mechanisms in tamoxifen resistance. We then investigated whether Mps1 regulates ERα transactivational activity. Luciferase reporter assay was performed by transfecting ERα into ERα negative 293T cells together with ERα responsive element reporter (ERE-Luc) and Mps1. As was shown, Mps1 strongly enhanced the transcriptional activity of ERα
in 293T cells. Moreover, the transcriptional activity of ERα was also significantly increased in ERα-positive MCF-7 regardless of the presence of 17β-estradiol (E2) (Figure 2B). In particular, expression of kinase inactive Mps1 D664A mutant failed to increase ERα transcriptional activity, indicating the indispensable role of Mps1 Kinase activity in the stimulatory effect of Mps1 (Figure 2C). A similar result was also achieved by using Mps1 inhibitor reversine (Figure 2C). Specifically, inhibition of Mps1 by Mps1 siRNA oligos reduced transcriptional activity of ERα induced by E2 treatment, which could be partially rescued by Mps1 reintroduction into siMps1 cells (Figure 2D). Consistent with this results, overexpression of WT Mps1, but not Mps1 D664A mutant, led to increased expression of ERα target genes catD and pS2 as shown by luciferase reporter assay (Figure 2E). Enhancement of catD, pS2, VEGFa, c-Myc and cyclinD1 expression by Mps1 overexpression were also observed (Figure 2F). Thus, Mps1 stimulates the transcriptional activity of ERα in its kinase-dependent manner.

**Mps1 interacts with ERα**

To determine the mechanism of the stimulatory role of Mps1 on ERα transactivational activity, we then try to evaluate whether Mps1 physically interacts with ERα. Specially, endogenous Mps1 and ERα formed a complex (Figure 3A). Co-immunoprecipitation experiments also showed that Myc-Mps1 associated with Flag-ERα (Figure 3B), and the interaction between Mps1 and ERα increased considerably in the presence of tamoxifen treatment (Figure 3C). Immunofluorescence staining showed that Mps1 colocalized with ERα mainly in the nucleus (Figure 3D). The interaction domains of ERα and Mps1 are AF1 and DNA binding domain (DBD) (Figure 3E).

**Mps1 mediates phosphorylation of ERα**

We next investigated whether ERα could be a new substrate for this serine/thrine kinase. In vitro kinase assay showed that ERα was highly phosphorylated in the reaction containing WT Mps1, but not Mps1 D664A mutant (Figure 4A). To further determine whether Mps1 phosphorylates ERα in vivo, immunoprecipitation was performed with anti-Ser/Thr antibody in 293T cells transfected co-transfected with Flag-ERα and either WT Mps1 or D664A mutant. Notably, phosphorylation of ERα was only induced by WT Mps1, whereas this increased phosphorylation was greatly reduced by reversine treatment or D664A mutant(Figure 4B-C).

To further analyze the potential phosphorylation site(s) of ERα by Mps1, Flag-tagged ERα coexpressed with Mps1 in HEK293 cells was subjected to mass spectrometry analysis. The results showed Ser559 and Thr224 of ERα were the two potential phosphorylation sites (Figure 4D-E). We then generated two ERα mutants in which the residues 559 and 224 were mutated to alanine individually. A sharp reduction of phosphorylation induction was observed in ERα S559A and T224A mutants compared to WT ERα respectively (Figure 4F-G). These results indicated that ERα-Ser559 and –Thr224 are phosphorylated by Mps1.

**Mps1 Increases ERα transcriptional activity primarily through phosphorylation of ERα at Ser559 or Thr224 Site**
We then determined whether the involvement of Mps1 on estrogen signaling depends on phosphorylation of ERα-Ser559 and -Thr224. The effect of Mps1 on ERα protein expression was first analyzed. Overexpression of Mps1 led to increased ERα expression (Figure 5A). In contrast, Mps1 D2664A mutant failed to upregulated ERα expression (Figure 5A). co-transfection of Mps1 significantly increased the protein level of WT ERα, but not that of ERα-Ser559 or –Thr224 mutant (Figure 5B). Consistently, Mps1 overexpression led to enhanced transcriptional activity of ERα in the presence of WT ERα but not ERα Ser559 or –Thr224 mutant (Figure 5C). Furthermore, the estimated half-life of WT ERα was significantly longer than that of ERαSer559 or –Thr224 (Figures 5D). These results suggested that the effect of Mps1 on ERα protein level and its transcriptional activity is mainly caused by phosphorylation on Ser559 or Thr224 sites.

Discussion

The phosphorylation of ERα has been shown to influence the ability to bind EREs and gene expression, elucidating the regulation mechanism of ERα phosphorylation may provides insights into potential adjuvant therapeutic target in breast cancer endocrine therapy\cite{19,20}. In this study, the important role of the Mps1 kinase protein in tamoxifen resistance in breast cancer was revealed. First, Mps1 interacted with ERα and mediates ERα phosphorylation at the Ser-559/Thr224 site. Second, ectopic Mps1 expression induced ERα transactivational activity, increased the expression of ERα target genes, and contribute to tamoxifen resistance in breast cancer cells. Third, both ERα S559A or T224A mutated failed to induce ERα transactivational activity\cite{21}. Therefore, phosphorylation regulation of ERα activity by Mps1 may contribute to tamoxifen resistance in breast cancer.

Phosphorylation of ERα occurs on multiple amino acid residues by several kinases\cite{22}. Modification of ERα S305 by PAK1 and TBK1 phosphorylation is associated with increased aggressiveness of tumors\cite{23}. In contrast, ERα S167 and/or S118 phosphorylation is often associated with good clinical outcome in patients with tamoxifen therapy\cite{24}. Here we show the importance of Mps1 kinase in ERα S559 and T224 phosphorylation, and that the half-life of WT ERαwas significantly longer than that of ERα S559A and T224A mutants, suggesting that these two phosphorylation sites protected ERα from degradation. Because administration of Mps1 kinase inhibitor together with tamoxifen achieved a synergistic effect on cancer cells proliferation, these two ERα phosphorylation may be associated with worse clinical outcome in patients who have breast cancer, which needs further investigation.

A large number of human cancers can tolerate with aneuploidy and chromosomal instability due to overexpressed SAC components including Mps1 kinase\cite{25}. Reduction in Mps1 levels or its activity in such tumors represents an effective strategy to target cancers with chromosomal instability\cite{26,27}. Several Mps1 inhibitors have been discovered and have undergone preclinical research\cite{28}. Here in our study, we demonstrate the importance of Mps1 expression on the responsiveness of breast cancer cells to tamoxifen, and targeting Mps1 might sensitize breast cancer cells to tamoxifen treatment. This sensitization is likely related to previously uncharacterized roles of Mps1 in regulating ERα signalling.
Therefore, Mps1 might form a unique therapeutic target for overcoming tamoxifen resistance in breast cancers.

**Materials And Methods**

**Plasmids and SiRNA.**

The reporter constructs ERE-Luc, catD-Luc, and pS2-Luc and the expression vectors for ERα, Flag-tagged ERα, HA-tagged ERα, and all Mps1 expression constructs were generated. The cDNA target sequence of siRNA for Mps1 is GCACGUGACUACUUU CAAAUU. Flag-tagged ERα mutants were generated by quick-change mutagenesis kit (Stratagene). Primers for mutations are:

S559A1: 5'-GGAGGGGCGAGCTGCTGAGGACG-3'

S559A2: 5'-CGTCTCCTCCACAGCTGCCCTCC-3'

T224A1: 5'-TATATGTGTCCAGCCGCCAACCAGTCACCATAC-3'

T224A2: 5'-ATGGTGACTGGTTGGCGGCTGGACACATATAC-3'

All plasmids were verified by restriction enzyme analysis and DNA sequencing.

**Real-time RT-PCR**

Real-time PCR was performed using PerfectStart® Green qPCR SuperMix (TransGen Biotech, AQ601-04) in triplicate and analyzed on an QuantStudio™ 3 Real-Time PCR System analyser (Thermo Fisher Scientific, A28136) as previously described. All real-time values were normalized to GAPDH. The primer sequences are:

for pS2:

5'-CTTCAGTGAGTCGCTGTTT-3' (forward)

5'-CCCAGGCAAGTCCA-3' (reverse);

for VEGFa:

5'-GCTACGACGCAATCGACC-3' (forward)

5'-CAGCTCCAGCCTCGTCA-3' (reverse);

for CyclinD1:

5'-CTGCGATCAACTTCTCG-3' (forward)

5'-GTCACACTTTGTCACTGG-3' (reverse);
for CathepsinD:

5’- CACAACCTACTGGCCGACGAG -3’ (forward)
5’- GATTCCAGAAACGGCCCACA -3’ (reverse);

for GAPDH:

5’-CATGTTCGTCATGGGTGTGAACCA-3’ (forward)
5’-AGTGATGGCATGGACTGTGGTCAT-3’ (reverse);

Cell Culture and Transfection

All cells were cultured at 37°C in 5% CO₂ incubator, 293T cells were cultured in DMEM(EallBio, 03.1002C) medium, and ZR-751 cells were cultured in RPMI1640(EallBio,03.4007C) medium. MCF-7 breast cancer cells were cultured in DMEM(EallBio, 03.1002C) medium containing 0.01mg/ mL insulin, all medium containing 10% FBS, and the medium was changed every 2 days. In the hormone treatment experiment, 0.1% ethanol containing 1 μM tamoxifen was added into the culture medium of experimental cells, and 0.1% ethanol was added into the culture medium of control cells.

Luciferase reporter assays

293T and MCF-7 cells were cultured in DMEM containing 10% FBS at 37 °C in a humidified atmosphere of 5% CO₂. For transfection, cells were seeded in 24-well plates containing phenol red-free DMEM medium supplemented with 2.5% charcoal dextran-treated FBS. The cells were transfected using Lipofectamine 2000 with 0.5 μg of ERE-LUC reporter plasmid, 50 ng of ERα expression vector and different amout of Mps1 or its mutants, and the respective empty vector was used to adjust the total amount of plasmid. Six hours later, the transfected cells were treated with 10 nM 17β-estradiol (E2) or 0.1% ethanol for 24 h, and then were harvested. Luciferase activities were determined according to the Firefly & Renilla Luciferase Reporter Assay Kit (Meilunbio,MA0518). All experiments were repeated at least three times with similar results

Cell Migration Assays

Cells were cultured in 6-well dishes and in DMEM medium containing 10% FBS. After cells adhesion, 95% confluence was achieved. A single layer of cells was wounded with the tip of a sterile pipette and a line was drawn on the diameter line of each hole to form a 1 mm cell-free path. Remove medium, with phosphate buffer (phosphatebuffersaline, PBS) gently cleaning the floating cells, after repeated 2-3 times, add 2 ml 10% FBS cultured MEM medium. The cells were fixed with 3.7% paraformaldehyde at the indicated times interval and photographed under a low power microscope to observe the healing of cell scratches

Western Blotting and Immunoprecipitation.
Cell extracts were prepared and immunoprecipitated were analyzed as previously described [29]. In brief, an aliquot of the total lysate [5% (vol/vol)] was included as a control for the interaction assay. Immunoprecipitation was performed with anti-Flag M2 Affinity Gel (A2220; Sigma–Aldrich), anti-Myc (A5598; Sigma–Aldrich), anti-ERα (catalogno. sc-7207; Santa Cruz Biotechnology), or anti-HA (H9658; Sigma–Aldrich) antibody. The antigen/antibody complexes were visualized by chemiluminescence.

**In Vitro Kinase Assay.**

293T cells were transfected with Flag-MPS1 or Flag-MPS1 (D2A) plasmid for 24 h. Cell lysates were prepared, and purified GST-ERα (2 μg) was mixed with the lysates in kinase buffer [20 mM Hepes (pH 7.5), 75 mM KCl, 10 mM MgCl2, and 10 Mm MnCl2] containing 2.5 mCi of [γ-32P]-ATP for 30 min at 37 °C. The reaction products were analyzed by SDS/PAGE and autoradiographed.

**Statistics**

Statistical analysis was performed using SPSS 17.0 (SPSS, Inc.) and R 2.13.0 (www.r-project.org). n = 3 independent experiments. All statistical tests were two-sided, and P values <0.05 were considered to be statistically significant.

**Declarations**

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**Author Contributions**

XL Y, CW W and YH T were involved in the conception and design of the study, and supervising the manuscript; XM Z in drafting the manuscript; XM Z, QG and YL Z in the revising the manuscript; XP M, CJ D, JL L, LF H and JY F in the acquisition, and analysis of the data; LM W, XP Y, YH Z, JF L, MY L, JL and HZ in the interpretation of the data. All the authors have discussed the results and reviewed the manuscript.

**Competing Interests Statement**

The authors have declared that no competing interests exist.

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**Figures**
Figure 1

Mps1 determines the sensitivity of breast cancer cells to tamoxifen. A. Crystal violet staining of six-well plate colony formation assays with 500 MCF7 cells transfected with siMps1 oligos or Flag-Mps1 in the presence or absence of tamoxifen (1 μM) for 14 days. B-C. Would healing assays in MCF7 cells transfected with Flag-Mps1 (B) or siMps1 oligos (C) in the presence or absence of tamoxifen (1 μM) for 24 hrs. D. Cell counting assay in MCF7 cells treated with the indicated concentrations of tamoxifen.
and/or Reversine continuing for 6 days (All groups compared with PBS control cells). E. Crystal violet staining of six-well plate colony formation assays with \( \approx 500 \) MCF7 or ZR-75-1 cells treated with tamoxifen (1 \( \mu M \)) and/or Reversine(2 \( \mu M \)) for 14 days.

**Figure 2**

Mps1 stimulates the transcriptional activity of ER\( \alpha \) (A-B) Luciferase assay in 293T cells (A) or MCF7 cells (B) transfected with the ERE-Luc reporter, ER\( \alpha \) and Mps1 with or without E2 for 24 hrs. (C) Luciferase
assay in MCF-7 cells transfected with ERE-Luc, Mps1 or Mps1 D664A with or without Reversine for 2 hrs. (D) Luciferase assay in MCF-7 cells transfected with Msp1 siRNA oligos (siMsp1) and a siRNA-resistant Msp1 (siMsp1/ Msp1) expression plasmid. (E) Luciferase assay in MCF-7 cells transfected with pS2-Luc or catD-Luc, Mps1 or Mps1 D664A. (F) qRT-PCR assay using the primers specific for ERα-responsive genes including cyclinD1, VEGFα, c-Myc, pS2 and catD in MCF-7 cells transfected with Flag-Mps1.

**Figure 3**
Mps1 interacts with ERα (A,C) Anti-Flag or anti-Mps1 immunoprecipitates were analyzed by immunoblotting with the indicated antibody in MCF-7 cells (A) or from MCF-7 cells treated with tamoxifen for the indicated times (C). (B) Anti-Flag immunoprecipitates were analyzed by immunoblotting with the indicated antibody in 293T cells co-transfected with Flag-ERα and Myc-Mps1 expression plasmids using the indicated antibodies. (D) Representative image of microscopy assay in 293T cells co-transfected with RFP-ERα and GFP-Mps1 expression plasmids. (E) Anti-Flag immunoprecipitates were analyzed by immunoblotting with the indicated antibody in 293T cells co-transfected with Myc-Mps1, Flag-ERα, or Flag-ERα mutants.
Figure 4

Mps1 mediates phosphorylation of ERα. A. Recombinant GST-ERα was incubated with Flag-Mps1 or Flag-Mps1 D664A immunoprecipitates from transfected 293T cells in the presence of [γ-32P]-ATP. The reaction products were analyzed by SDS-PAGE and autoradiography. (B) Anti-Flag immunoprecipitates were analyzed by immunoblotting with anti-p-Ser or anti-Flag antibody in 293T cells co-transfected with the indicated plasmids. (C-D) MS analyses on ERα recovered from 293T cells transfected with Mps1. (E-
F) Anti-Flag immunoprecipitates were analyzed by immunoblotting with anti-p-Ser or anti-Flag antibody in 293T cells co-transfected with Flag-ERα or its mutants.

**Figure 5**

**A**

| Flag-ERα | Myc-Mps1(μg) | Myc-Mps1-D664A |
|----------|---------------|-----------------|
| 0        | 0.2           | 0.5            |
| 1.0      | -             | -              |
| 0.2      | -             | +              |

**B**

| Flag-vector | Flag-ERα | Flag-ERα(S559A) | Flag-ERα(T224A) |
|-------------|----------|-----------------|-----------------|
| +           | +        | +               | +               |
| -           | -        | -               | -               |
| +           | -        | +               | +               |
| -           | -        | -               | -               |
| +           | +        | +               | +               |
| -           | -        | -               | -               |

**C**

![Graph showing ERE activation](image)

**D**

| Flag-ERα | Flag-ERα(S559A) | Flag-ERα(T224A) |
|----------|-----------------|-----------------|
| 0        | 3               | 9               |
| 6        | 9               | 12              |
| 12       | 24              | 24              |
| CHX(h)   | CHX(h)          | CHX(h)          |
| 0        | 3               | 6               |
| 9        | 12              | 24              |
| IB: Flag | IB: Flag        | IB: α-Tubulin   |

**Figure 5**

Mps1 Increases ERα transcriptional activity primarily through phosphorylation of ERα at Ser559 or Thr224 Site (A) Immunoblotting assay in 293T cells transfected with Flag-ERα and the increasing doses of Flag-Mps1 or Flag-Mps1 D664A. (B) Immunoblotting assay in 293T cells transfected with Myc-Mps1, Flag-ERα or its mutants. (C) Luciferase assay in 293T cells transfected with the ERE-Luc reporter, Myc-Mps1, Flag-ERα or its mutants. (D) 293T cells were transfected with the expression vector encoding Flag-
ER$\alpha$ or its mutant. After 24 hrs, both cells were treated with cycloheximide (50 μM), the level of ER$\alpha$ was monitored by immunoblotting using anti-HA antibody. α-Tubulin was used as equal loading control.