FULL LENGTH ARTICLE

Effect of Sirtuin 1 inhibition on matrix metalloproteinase 2 and Forkhead box O3a expression in breast cancer cells

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Abstract
Breast cancer is the most common invasive cancer in women worldwide. Sirtuin 1 (SIRT1) has recently been shown to have implications in regulating cancer cell growth and apoptosis. SIRT1 regulates Forkhead box O3a (FOXO3a) by both inhibiting FOXO3-induced apoptosis and potentiating the ability of FOXO3a to resist oxidative stress. Matrix metalloproteinase 2 (MMP2) participates in tumor invasion and metastasis by degrading extracellular matrix. SIRT1 up regulates MMP2 expression by its deacetylation activity. This study aimed to investigate the expression of SIRT1, FOXO3a and MMP2 in breast tissues of women with breast cancer. In addition, the effect of SIRT1 inhibition on both FOXO3a and MMP2 expression in breast cancer (MCF-7) cells was assessed. The expression levels of SIRT1, FOXO3a and MMP2 in the breast tissues were determined by real-time PCR in 60 patients with malignant tumor and in 24 patients with benign tumors. After SIRT1 inhibition, protein levels of SIRT1 and FOXO3a were assessed by Western Blot and levels of MMP2 by ELISA in MCF-7 cells. The expression levels of SIRT1, FOXO3a and MMP2 were significantly higher in breast cancer tissues compared to in benign breast tumor and adjacent normal tissues. SIRT1, MMP2 and FOXO3a expression were associated directly with each other. SIRT1 inhibition suppresses MMP2 and FOXO3a expression compared to control MCF7. Sirtinol (SIRT1 inhibitor) effectively induced inhibition of MMP2 and FOXO3a expression in MCF-7 cells, indicating the promising therapeutic strategy of targeting SIRT1 for breast cancer.

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The effects of SIRT1 on FOXO function are complex and vary depending upon the target genes of FOXO. It has been found that SIRT1 upregulated the transcription of FOXO target genes involved in cell survival under stress; however, it downregulated the transcription of genes associated with cell death. Thus, SIRT1 appeared to shift the FOXO-dependent response far from cell death toward stress resistance. This could be explicated by the concept that acetylation/deacetylation of FOXO protein may switch target specificity.3

Matrix metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases that have a pivotal role in promoting cancer cell invasion through the degradation of extracellular matrix. They have been found in greater amounts and higher activity inside and around malignant cancers than in normal, benign, or premalignant tissues.5 MMPs can present in both inactive pro-enzymes and active enzymes forms. The mechanism of activation in vivo is still unknown but may be mediated by proteolytic activity of other MMPs and/or serine proteases.10

One of the MMPs implicated in cancer invasion is MMP-2 which also known as gelatinase A. This MMP is thought to mediate invasion and metastasis through the degradation of type IV collagen, the main component of basement membranes which induces angiogenesis.11 MMP-2 expression and activity are regulated by SIRT1 at posttranslational level. It has been found that the up regulation of MMP-2 expression by SIRT1 is mediated by its deacetylation activity. However, SIRT1 knockdown reduces MMP-2 expression through decreasing its protein stability.12

The present study aimed to investigate SIRT1, FOXO3a and MMP2 gene expression in breast cancer, benign breast tumor and adjacent normal tissues as well as to study the correlations of SIRT1, FOXO3a and MMP2 with clinicopathological and biochemical parameters. Additionally, we aimed to assess the effect of SIRT1 inhibition on both FOXO3a and MMP2 gene expression in breast (MCF-7) human cancer cells.

Materials and methods

Ethical approval

The study was approved by the Institutional Review Board (IRB) of the National Cancer Institute (NCI), Cairo University and was conducted according to the rules of Helsinki declaration for human studies. A Written informed consent was obtained from all patients.

Patients

The current study enrolled 84 female patients recruited from National Cancer Institute, Cairo, Egypt. They were divided into two groups; the first group included 60 patients who have newly diagnosed breast cancer while the second group included 24 patients who have newly diagnosed benign breast tumor. The classification of tumor and its stage were performed according to the international union against cancer (Tumor—Node—Metastasis) classification. The breast cancer histopathology was done using biopsy from tumor cancer tissues and from adjacent normal tissue. Also, the benign breast tumor histopathology was implemented using tissue biopsy of mammary tumor or after surgery. Furthermore, three tissue cores were taken from all breast lesions, one of them stored in RNA lysis solution at −80 °C for genetic processing of SIRT1, FOXO3a and MMP2
genes and the other two cores stored within formalin 10% for histopathological and hormonal receptors assessment.

Biochemical analysis

Three ml of peripheral blood sample were collected for assessment of serum cancer antigen 15.3 (CA 15.3) and serum carcinoembryonic antigen (CEA) following standard laboratory methods.

Real-time quantitative analysis for SIRT1, FOXO3a and MMP2 gene expression

Total RNA was extracted from tissue homogenate using SV Total RNA Isolation System (Promega, Madison, WI, USA) according to manufacturer’s instruction. Complementary DNA (cDNA) was synthesized from 1 µg RNA using SuperScript III First-Strand Synthesis System as described in the manufacturer’s protocol (#K1621, Fermentas, Waltham, MA, USA). Real-time quantitative PCR amplification and analysis were performed using an Applied Biosysyem with software version 3.1 (StepOne™, USA). The reaction contained SYBR Green Master Mix (Applied Biosystems), gene-specific primer pairs designed with Gene Runner Software (Hasting Software, Inc., Hasting, NY) from RNA sequences from Gen Bank (SIRT1: Forward primer 5′-AGAGCGCTCAACTGCAAGCTCTAG-3′, Reverse primer 5′-GCACATGATGTTGGACAC-3′, FOXO3a: Forward primer 5′-CGACTATGCAAGGTCCAGGTTG-3′, Reverse primer 5′-CGACATGCACTGACAGGTTG-3′, MMP2: Forward primer 5′-GCCCTCTGACTCTCAGTAG-3′, Reverse primer 5′-GCCCTCTGACTCTCAGTAG-3′, GAPDH: Forward primer 5′-CCAGTTGTTGCTCTGACTTT-3′, Reverse primer 5′-GGGGCTGCGGAAGGAGG-3′). All primer sets had a calculated annealing temperature of 60°C. Real-time quantitative PCR was performed in a 25-µl reaction volume consisting of 2X SYBR Green PCR Master Mix, 900 nM of each primer and 2 µl of cDNA. Amplification conditions were: 2 min at 50°C, 10 min at 95°C and 40 cycles of denaturation for 15 s and annealing/extension at 60°C for 10 min. Data from real-time assays were calculated using the v1.7 Sequence Detection Software from PE Biosystems (Foster City, CA). All values were normalized to the GAPDH which was used as the endogenous control (reference gene). Relative quantifications were calculated using the 2^ΔΔCt method.13

Cultures of human breast cancer (MCF7) cells

Breast (MCF-7) human cancer cells was obtained from the tissue culture unit of the Holding Company for Biological Products and Vaccines (VACSERA), Giza, Egypt and supplied through the American Type Culture Collection (ATCC; Virginia, USA) and were grown in a sterile 50 cm² tissue culture flask in complete medium containing Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% phosphate-buffered saline (PBS) and 1% penicillin-streptomycin (100 units/mL penicillin and 100 µg/mL streptomycin). All cells were incubated in a humidified atmosphere incubator containing 5% CO₂ at 37°C. Cells were cultured to 100% confluence. Cells were passaged using trypsin-EDTA. The cultured MCF7 cells were divided into two groups: 1st MCF7 cells as control cells, 2nd MCF7 cells were treated with sirtinol (SIRT1 inhibitor) which was purchased from Sigma; dissolved in dimethyl sulfoxide (DMSO) at a dose 120 µM for 24 h, then the media were collected and centrifuged at 10,000 rpm for 20 min, the supernatant was kept frozen at −80°C till analysis of MMP2 by ELISA. MCF7 cells were harvested for assessment of protein expression of SIRT1 and FOXO3a by western blot.

Western blot analysis of SIRT1 and FOXO3a

(using V3 Western Workflow™ Complete System, Bio-Rad Hercules, CA, USA) Cells were washed with ice-cold PBS, trypsinized, and collected by centrifugation. Protein were extracted from cell lysates using ice-cold radio-immunoprecipitation assay (RIPA) lysis buffer PL005 was provided by Bio BASIC INC (Markham Ontario L3R 8T4 Canada) (50 mM Tris HCl, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS (sodium dodecyl sulfate)) supplemented with phosphatase and protease inhibitors, then centrifugation at 12,000 rpm for 20 min at 4°C. The protein concentration for each sample was determined using Bradford assay. Equal amounts of protein (20–30 µg of total protein) and 2X Laemmli buffer were heated at 70°C for 5–10 min and separated by SDS/polyacrylamide gel electrophoresis (10% acrylamide gel) using a Bio-Rad Mini-Protein II system. The protein was transferred to polyvinylidene difluoride (PVDF) membranes (Pierce, Rockford, IL, USA) with a Bio-Rad Trans-Blot system (TGX Stain-Free FastCast™ Acrylamide Kit which was provided by Bio-Rad Laboratories, TNC, USA). After transfer, the membranes were washed with Tris Buffer Saline (TBS) and blocked for 1 h at room temperature with 5% (w/v) skimmed milk powder in TBS. The manufacturer’s instructions were followed for the primary antibody reactions. Following blocking, the blots were developed using antibodies for SIRT1, FOXO3a and β-actin supplied by (Thermoscientific, Rockford, Illinois, USA) incubated overnight at pH 7.6 at 4°C with gentle shaking. After washing, peroxidase-labeled secondary antibodies were added, and the membranes were incubated at 37°C for 1 h then washed with TBS 5 times for 5 min. Chemiluminescence substrate (Clarity Western ECL Substrate BIO-RAD, USA) was applied to the blot according to manufacturer’s recommendation. Band intensity was analyzed by ChemiDocTM imaging system with Image LabTM software version 5.1 (Bio-Rad Laboratories Inc., Hercules, CA, USA). The results were expressed as arbitrary units after normalization for β-actin protein expression.

Assessment of MMP-2

(30,000,06130): Cell culture supernatant was centrifuged for 20 min to remove insoluble impurity and cell debris at 1000×g at 2–8°C. The clear supernatant was used for assessment of MMP2 immediately.

Statistical analysis

Analysis of more than two variables was done by One-Way ANOVA followed by Tukey’s comparison test. Two sample t
test was used for comparison of two quantitative variables. Simple linear correlation (Pearson’s correlation) was also carried out. Data are expressed as mean ± SD using Graphpad prism version 5.0. The statistical significance was set as $P < 0.05$.

**Results**

**Patient features**

The study included 60 patients with primary malignant breast cancer with mean age of 52.18 ± 12.17 years and 24 patients with benign breast tumor with mean age of 45.55 ± 10.14 years. The clinical features of the patients, including histology, histopathological grade of the tumor, axillary lymph node status, tumor size and clinical grade (TNM stage) were shown in Table 1. The majority of the patients were invasive duct carcinoma (N = 49, 81.7%) and moderately differentiated in histologic grade (N = 46, 76.7%), and large number of patients had no lymph node metastasis (N = 35, 58.33%). Most of the cases were positive ER (N = 47, 78.33%), PR (N = 45, 75%) and negative HER2 (N = 42, 70%). The percentage of tumors at stage 2 and 3 at the time of diagnosis was 65% and 35%, respectively. Histology of benign breast tumor showed that 70.7% (N = 17) of patients were fibroadenoma, 12.5% (N = 3) were fibrocystic mastopathy, 4.2% (N = 1) were spindle cell tumor, 4.2% (N = 1) were granulomatous mastitis, 4.2% (N = 1) were fat necrosis and 4.2% (N = 1) were fibro-epithelial tumor.

**Serum levels of tumor markers**

The levels of CA 15.3 and CEA were significantly higher in malignant breast cancer group (96.7 ± 13.04, 12.2 ± 3.39, respectively) compared to benign tumor group (16.76 ± 4.9, 2.29 ± 0.7, respectively) at $P < 0.001$.

**Tissue levels of SIRT1, MMP2 and FOXO3a**

It has been shown that SIRT1, MMP2 and FOXO3a were significantly overexpressed in breast cancer tissues (10.73 ± 1.32, 13.18 ± 1.49, 14.21 ± 2.06, respectively) compared to in benign breast tumor (2.47 ± 1.15, 2.54 ± 0.6, 4.46 ± 0.49, respectively) and adjacent normal tissues (1.012 ± 0.044, 1.015 ± 0.033, 1.00 ± 0.013, respectively) at $P < 0.001$ (Fig. 1).

**Effect of SIRT1 inhibition on MMP2 and FOXO3a expression**

In order to study the regulation of SIRT1 on MMP2 and FOXO3a expression, we treated breast cancer cell line (MCF7) with sirtinol (SIRT1 inhibitor), and found that SIRT1 inhibition suppresses FOXO3a expression (0.27 ± 0.029) and decrease MMP2 level (4.22 ± 0.334) compared to control MCF7 (1.03 ± 0.028, 16.23 ± 1.01, respectively) at $P < 0.001$ as shown in Figs. 2 and 3.

**Pearson’s correlation analysis**

As shown in Table 2, there was a significant inverse correlation between MMP2, FOXO3a expression with age and CEA ($P < 0.001$, 0.01 and 0.02, 0.001, respectively). SIRT1 was associated directly with tumor grade and stage ($P$: 0.005 and 0.02, respectively). Also, there was a significant direct correlation between MMP2, FOXO3a expression and tumor stage ($P$: 0.01 and 0.03, respectively). There was a

![Figure 1](image-url) **Expression of SIRT1, MMP2 and FOXO3a in breast tissues of the studied groups. Parameters were presented on the charts as means ± SD. SIRT1: sirtuin 1, FOXO3a: forkhead box O3a, MMP2: matrix metalloproteinase 2. a: significant from normal tissues, b: significant from benign tissues.**
significant inverse correlation between SIRT1, FOXO3a expression with CA 15.3 (P: 0.03 and 0.002, respectively). SIRT1, MMP2 and FOXO3a were associated directly with lymph node status (P: <0.001, <0.001 and 0.01, respectively). Regarding correlation between SIRT1, MMP2 and FOXO3a expression, there was a significant direct correlation between them (P < 0.001).

**Discussion**

Breast cancer is one of the most frequent cancers and it is the leading cause of death related to cancer among women worldwide. Death from breast cancer results from distant metastasis rather than primary cancer. It has been suggested that SIRT1 has a promoting function in tumor development and progression through the deacetylation of some key cell cycle and apoptosis regulatory proteins like p53 and FOXO leading to suppression of their function.

The present study showed that there was an extremely high frequency of overexpressed SIRT1 in breast malignant tumor tissues compared to their paired normal tissues and to benign tumor tissues. The expression of SIRT1 in breast cancer tissues is contentious. In accordance to our results, Derr et al., Kuo et al. and Sung et al. demonstrated an overexpression of the SIRT1 in breast cancer tissue than in normal tissue. In addition, several reports have demonstrated that overexpression of SIRT1 is not exclusively present in breast cancer tissue but also was observed in other cancer tissue, including prostate cancer, lung cancer, gastric cancer, and hepatocellular carcinoma.

On contrary to our results, Cao et al. and Wang et al. found a significant lower expression of SIRT1 in breast cancer tissue than in normal tissue. They reported that SIRT1 has an antitumor potential. Thus, SIRT1 may act as a tumor suppressor through its role in DNA damage repair and maintaining genome integrity. Until now, the definite role of SIRT1 is controversial.

Regarding MMP2 and FOXO3a, our results revealed a significant overexpression in breast cancer tissues compared to in benign breast tumor and adjacent normal tissues. In agreement with these results, other studies by Mahmood et al., Radenkovic et al., Sil et al. and Sullu et al. found that MMP2 expression was increased in breast cancer tissues as compared to that of benign tumor and adjacent normal tissues. On the other hand, Jiang et al. found that only 37% of breast cancer tissue samples expressing high level of FOXO3a. Furthermore, Chen et al.
and Lam et al. revealed that FOXO3a has been shown to be deregulated in breast cancer. In the present study, SIRT1 was associated directly with tumor grade, stage and lymph node status. These finding came in agreement with Wu et al. who found that the expression of SIRT1 was significantly correlated with tumor stage and lymph node status.

In this study, both of MMP2 and FOXO3a were significantly correlated directly with tumor stage and lymph node status which came in accordance with other studies that found a significant correlation between MMP2 and both tumor stage and lymph node metastasis. Additionally, Jia et al. found that FOXO3a expression was strongly associated with axillary lymph node status and TNM stage. Furthermore, it has been revealed that nuclear FOXO3a was associated with lymph node metastasis and poor survival in invasive ductal breast carcinoma.

Regarding correlation between SIRT1, MMP2 and FOXO3a expression, there was a significant direct correlation between them. To the best of our knowledge this is the first study assessing the effect of SIRT1 inhibition on both MMP2 and FOXO3a in breast cancer. In order to study the effect of SIRT1 on MMP2 and FOXO3a expression, the breast cancer cell line (MCF7) was treated by sirtinol (SIRT1 inhibitor). We found that SIRT1 inhibition suppresses MMP2 and FOXO3a expression compared to control MCF7. These finding came in agreement with Lovaa et al. who found that SIRT1 inhibition using sirtinol suppressed MMP2 expression in both LNCaP and PC3 prostate cancer cells. Furthermore, Kuo et al. revealed that the cell viability was alleviated by sirtinol in a time- and dose-dependent manner in MCF-7. In addition, Grbesa et al. found that SIRT1 inhibition via siRNA suppressed cell growth in lung cancer cell lines. These results suggested an association of SIRT1 expression with breast cancer development and pointed out the SIRT1 role in cancer cell growth.

Regarding the effect of SIRT1 on MMP2, SIRT1 has been reported as a positive regulator of MMP2 activity by promoting its expression, stability and activity. MMP2 has been found to have potential roles in intravasation and metastasis sustaining neovasculature not solely by stimulating angiogenic factors like vascular endothelial growth factor, but also through proteolytic remodeling activity of the tumor matrix. Taken together, SIRT1 inhibition suppresses MMP2 expression and as a result can decrease cancer invasion. Thus, SIRT1 inhibition may represent a potential therapeutic strategy for breast cancer.

Regarding the effect of SIRT1 on FOXO3a, it has been demonstrated that SIRT1 regulates FOXO3 by both inhibiting FOXO3-induced apoptosis and potentiating the ability of FOXO3 to resist oxidative stress. The effects of SIRT1 on FOXO vary depending on the FOXO target genes. SIRT1 potentiates the expression of FOXO target genes involved in stress resistance, but on the other hand decreases the transcription of genes involved in apoptosis leading to shift the FOXOs-dependent response away from apoptosis and toward stress resistance. Moreover, it has been shown that the inhibition of SIRT1 enhances the apoptosis triggered by oxidative stress. Thus, SIRT1 inhibition may contribute to the treatment of cancer combined with reactive oxygen species (ROS)-generating anti-cancer drugs.

**Conclusion**

SIRT1 inhibition decreased MMP2 and FOXO3a expression in MCF-7 cells, so it may attenuate or dampen cancer invasion and increase tumor cell apoptosis, pointing out the potential role of SIRT1 as promising therapeutic target for breast cancer.

**Conflict of interest**

No potential conflict of interest was reported by the authors.

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