Article published online: 2022-07-07

ARTICLE

Metformin Treatment Decreases mTOR mRNA Level in MCF-7 Breast Cancer Cells

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Published: 01 July 2016
Ibnosina J Med BS 2016;8(4):99-108
Received: 18 May 2016
Accepted: 19 June 2016
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Abstract

Besides the involvement of mTOR activity in several cancer conditions, evidence exists that increased total mTOR protein level might be linked to some cancer conditions such as colorectal carcinoma. The increase in total mTOR protein level in colon cancer was found to be associated with enhanced tumor progression and poor prognosis. Total mTOR protein level is elevated in breast cancer cells compared to their nonmalignant counterparts. High mTOR protein level in breast cancer cells could be attributed to decreased mTOR protein degradation, increased mTOR protein expression, or both. Increased protein expression may involve an increase in the gene expression.

Here, we investigated the possibility of increased *MTOR* gene expression as a potential underlying cause of the elevated total mTOR protein in breast cancer cells. Our results suggest that transcription of *MTOR* gene is increased in the estrogen receptor positive (ER+) MCF-7 breast cancer cells compared to other breast cell lines. DNA sequencing of the *MTOR* promoter identified sequence variations in MCF-7 cells, which could be potentially involved in upregulation of mTOR expression. Among these variations is a truncation of guanine thymine dinucleotide $(GT)_n$ repeat region in MCF-7 cells, which might be possibly implicated in the elevated transcription of *MTOR* gene in these cells. Moreover, our results revealed that metformin treatment, profoundly decreased mTOR mRNA levels in MCF-7 breast cancer cells.

In conclusion, unraveling the potential mechanisms involved in the regulation of mTOR expression in breast cancer cells could provide an avenue for optimizing the efficacy of breast cancer treatment regimens.

Key words: Breast cancer, metformin, *MTOR* gene expression.

Introduction

Mammalian target of rapamycin (mTOR) is a conserved serine/threonine kinase ubiquitously expressed in the cells to regulate growth and metabolism (1-3). It is a convergence point of several intracellular and extracellular stimuli to control various cellular processes. Thus, aberrant regulation of mTOR pathway results in a group of disease states, including type 2 diabetes, neurodegenerative diseases and cancer (4). Activated mTOR pathway is known to mediate many cellular functions, which promote invasiveness characteristics of a tumor such as proliferation, migration, and survival (5,6). In many types of cancer, including breast cancer (7), hyperactivation of mTOR signaling has been associated with aggressive tumor growth (8), whereas mTOR inhibition has been shown to sensitize breast cancer cells to the cytotoxic effects of chemotherapy *in vitro* (9) as well as to hormonal treatment (10).

Currently, mTOR inhibition is being extensively investigated for its potential anti-neoplastic effects. There are multiple mTOR inhibitors with different mechanisms of action (11). Generally, mTOR inhibitors exert their inhibitory effects on mTOR activity either allosterically by direct interfering with mTOR complex formation or catalytically by preventing mTOR phosphorylation (12). Rapamycin and its analogs (rapalogs) are highly specific inhibitors of mTOR. These are currently being evaluated as anticancer agents in numerous clinical trials (11). Rapamycin is an allosteric mTOR inhibitor, while the small molecule, PP242, inhibits mTOR activity catalytically by competition with ATP (12). Rapamycin inhibits the growth of a variety of epithelial cancers including those of the kidney, breast, and lung (13). However, toxicity is a limiting factor that precludes the use of high doses of rapalogs in combinatorial treatment for breast cancer (11). Inhibition of mTOR can also be achieved by the increase in AMP/ATP ratio induced by agents such as metformin (14). This anti-diabetic biguanide has recently received a huge attention for its promising anti-cancer effects (15). Similar to the direct mTOR inhibitors, metformin inhibits the phosphorylation and activation of mTOR and its downstream target P70-S6K (13). Metformin acts as a growth inhibitor for MCF-7 human breast cancer cells through the upregulation of AMP kinase (AMPK) activity (16). There is evidence that metformin also exerts an AMPK-independent inhibitory effects on the mTOR activity (14, 17) as well as on the cell cycle (17). The degree of inhibition of mTOR activity by metformin and rapamycin is comparable, but their effects on the cells proliferation, and viability are markedly different, which emphasizes the advantage of using metformin as an anticancer treatment (18). The concentrations of metformin required to achieve such beneficial effects are generally high, particularly that reaching the required availability of metformin at the peripheral tissues including the breast tissue requires the use of high doses. However, use of high concentrations of metformin is generally tolerable (19) compared to other mTOR inhibitors which are also required in high doses to achieve better antitumor effects. The relatively safe profile of metformin makes it a promising agent for treatment of breast cancer. However, the molecular basis of its beneficial effects in breast cancer is still far from being fully elucidated.

Total mTOR protein level is high in some cancers such as colorectal cancer and it correlates directly with the tumor stage (20), but the status of total mTOR protein and its impact in breast cancer cells are not well delineated. We have previously reported that total mTOR protein level is elevated in breast cancer cells compared to their nonmalignant counterparts. One of the potential causes underlying the high mTOR protein level in breast cancer cells is the increased expression of *MTOR* gene; however, the mechanisms regulating the transcription of *MTOR* gene are still largely unknown.

Gene regulation, generally, involves multiple steps of gene transcription, mRNA processing, and finally translation into proteins. Transcription is the first and the most common step in controlling a gene expression (21). Gene promoters usually contain binding sites which act as enhancers or silencers of the gene expression. Tandem repeats are made of two or more contiguous nucleotides scattered all over the genome sequence (21) and they are usually prone to polymorphism (22) repeats are the most common microsatellite in mammals (23). Presence of these tandem repeats in the promoter of a gene is of special importance as potential regulatory elements of the gene transcription (22). Therefore, variations in these repeats might have functional significance in the gene regulation and thereof clinical correlations. For instance, $(GT)_n$ polymorphism in the promoter region of *HMOX-1* gene has been shown to be associated with susceptibility to ischemic events (24). Moreover, variations of $(GT)_n$ dinucleotide region length in the first intron of *PIK3CA* gene has been linked to the risk of breast cancer (25) and colorectal cancer (26)

Materials and Methods

Cell lines and reagents

MCF-10A, MCF-7, and MDA-MB-231 breast cell lines were obtained from the ATCC, cultured, and stored following ATCC protocol of authentication by short terminal repeat analysis. The cells were grown in Dulbecco's modified Eagle's medium containing high glucose (4.5 g/l) (DMEM; Gibco Life Technologies, Carlsbad, CA, USA) and supplemented with 7% fetal bovine serum (Harlan Bioproducts for Science, Inc., Indianapolis, USA). Chymostatin, leupeptin, antipain, pepstatin, benzamidine, PMSF, DTT, insulin, metformin, rapamycin were purchased (Sigma Chemical Co, St. Louis, MO, USA).

RNA isolation and semi-quantitative RT-PCR.

Total RNA was isolated from proliferating MCF-10A, MCF-7, and MDA-MB-231 breast cell lines using the guanidinium thiocyanate method- according to the described protocol (28). Relative expression levels of mTOR mRNA were determined by semi-quantitative RT-PCR of an amplicon of around 361bp using gene-specific primers. mTOR Forward Primer: 5-'CCACTGTGCGGATCATTTC-3' (SENCE STRAND) and mTOR Reverse Primer: 5'-CTGGATGAGCATCTTGCG-3' (SENCE STRAND). The cDNAs were prepared by reverse transcription from 0.5μg of total RNA using TaqMan reverse transcription reagents and analyzed for mTOR and GAPDH according to the manufacturer's protocol (Applied Biosystems, Invitrogen). Expression levels were normalized against GAPDH. 50 μg of each sample of RNA was fractionated in a 1% agarose gel. Band densitometry was measured by AlphaView imaging software, FluorChem Q system, ProteinSimple.

DNA extraction and sequencing

Normal human genomic DNA was obtained from (Promega Corporation, Madison WI, USA). Genomic DNA was isolated and purified from MCF-10A, MCF-7, and MDA-MB-231 cells lines- by a method adopted from the protocol (28). 0.5 μg of the DNA was amplified with PCR using (GoTaq Flexi DNA Polymerase; Promega) and primers specific to *MTOR* promoter flanking the $(GT)_n$ repeat region. Samples were then submitted for Sanger sequencing in the DNA Core Facility of the University of

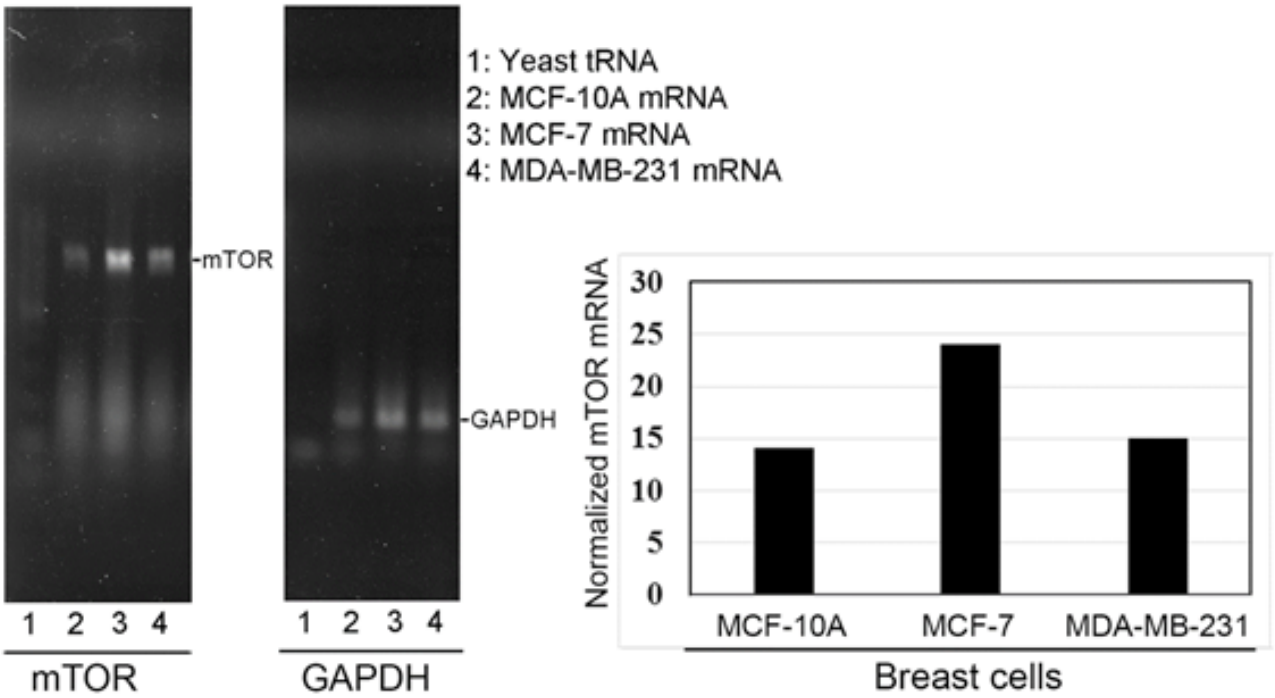

Figure 1. mTOR mRNA levels in noncancerous and cancerous breast cell lines. Total RNA of untreated (control) MCF-10A, MCF-7, MDA-MB-231 breast cell lines. Both the noncancerous as well as the cancerous breast cell lines were subjected to semi-quantitative RT-PCR analysis with primers specific for *MTOR*. The results were normalized to the level of GAPDH mRNA in each sample and yeast tRNA was used as control.

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Results

mTOR mRNA in MCF-7 breast cancer cells vs normal breast cells

We have shown previously that total mTOR protein level is higher in breast cancer cells, particularly in the ER+ MCF-7 cells, compared to their noncancerous counterparts, which correlates positively with an increased mTOR activity in these cells. Increased gene transcription results in an increase of mRNA level of the gene. Therefore, our hypothesis was that transcription of *MTOR* gene could be potentially one of the reasons of elevated mTOR protein level in the breast cancer cells. To investigate the possibility of increased transcription of *MTOR* gene in the breast cancer cells, we assessed the mRNA level of mTOR in cancerous and noncancerous breast cell lines. Consistent with our previous results, semi-quantitative RT-PCR assay revealed that MCF-7 breast cancer cells have higher level of mTOR mRNA as shown in Figure 1, lane 3. In contrast, the triple negative breast cancer cells, MDA-MB-231, did not show a significant difference in mTOR mRNA level from the noncancerous MCF-10A cells as shown in figure 1, lane 4 and lane 2, respectively. Bar graph representation shows the densitometry of mRNA in the breast cell lines following normalization for GAPDH mRNA level.

Identification of sequence variations in MTOR promoter of MCF-7 cells

To investigate a potential cause for the apparently increased *MTOR* gene transcription in MCF-7 breast cancer cells, we sequenced 1.9-kb DNA fragment of the promoter region of human *MTOR* gene. We submitted our sequencing results to the National Center for Biotechnology Information (NCBI-BankIt) and it was deposited under the accession number KJ399980 (29). We scanned the *MTOR* promoter obtained from normal human genomic DNA for elements which could serve as binding sites for transcription factors

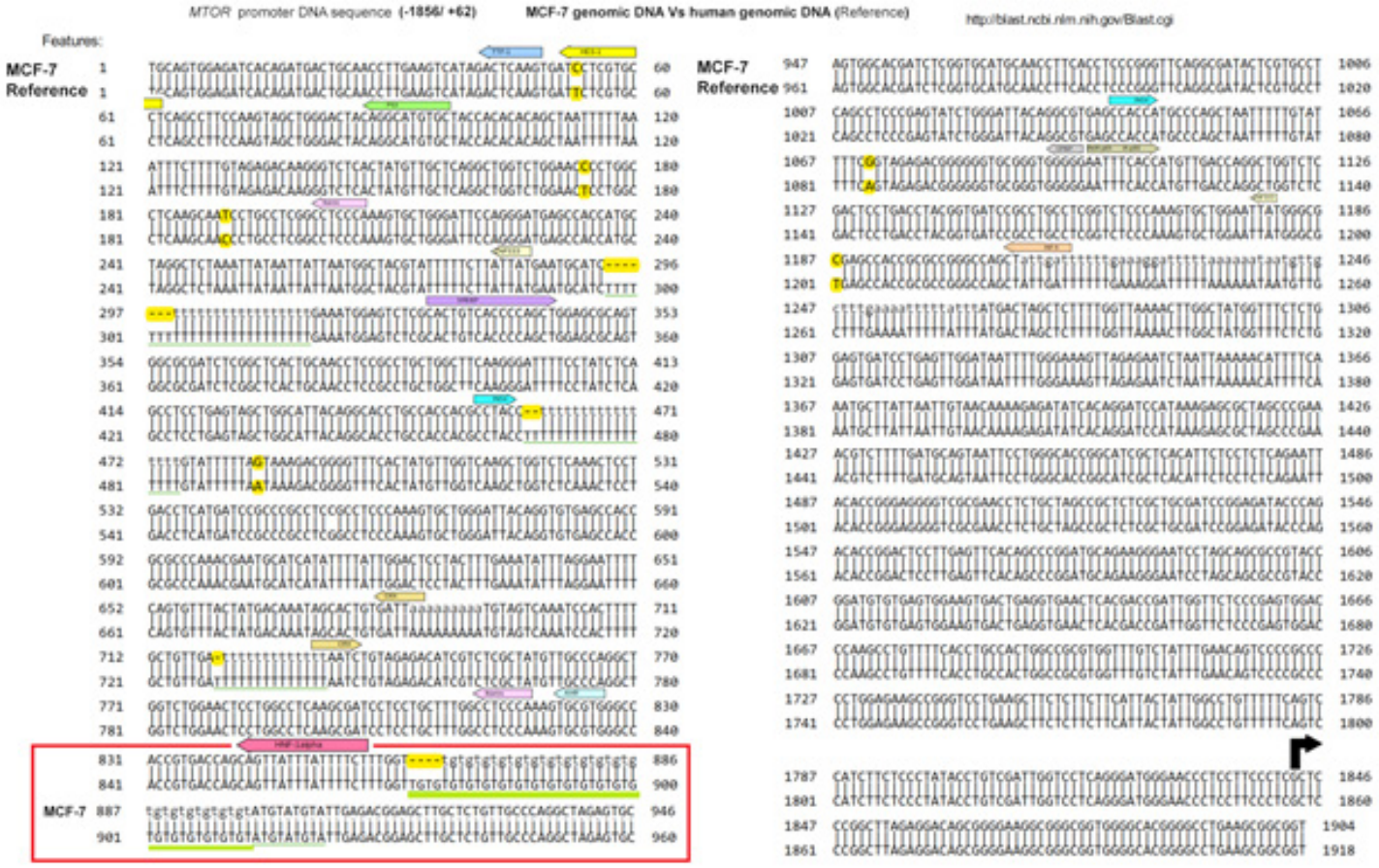

Figure 2. Sequence variation of MTOR promoter region in MCF-7 breast cancer cells. DNA sequences of 1.9 kb (-1856/ + 62) of MTOR prompter region from human genomic DNA (top line) and from genomic DNA of MCF-7 breast cancer

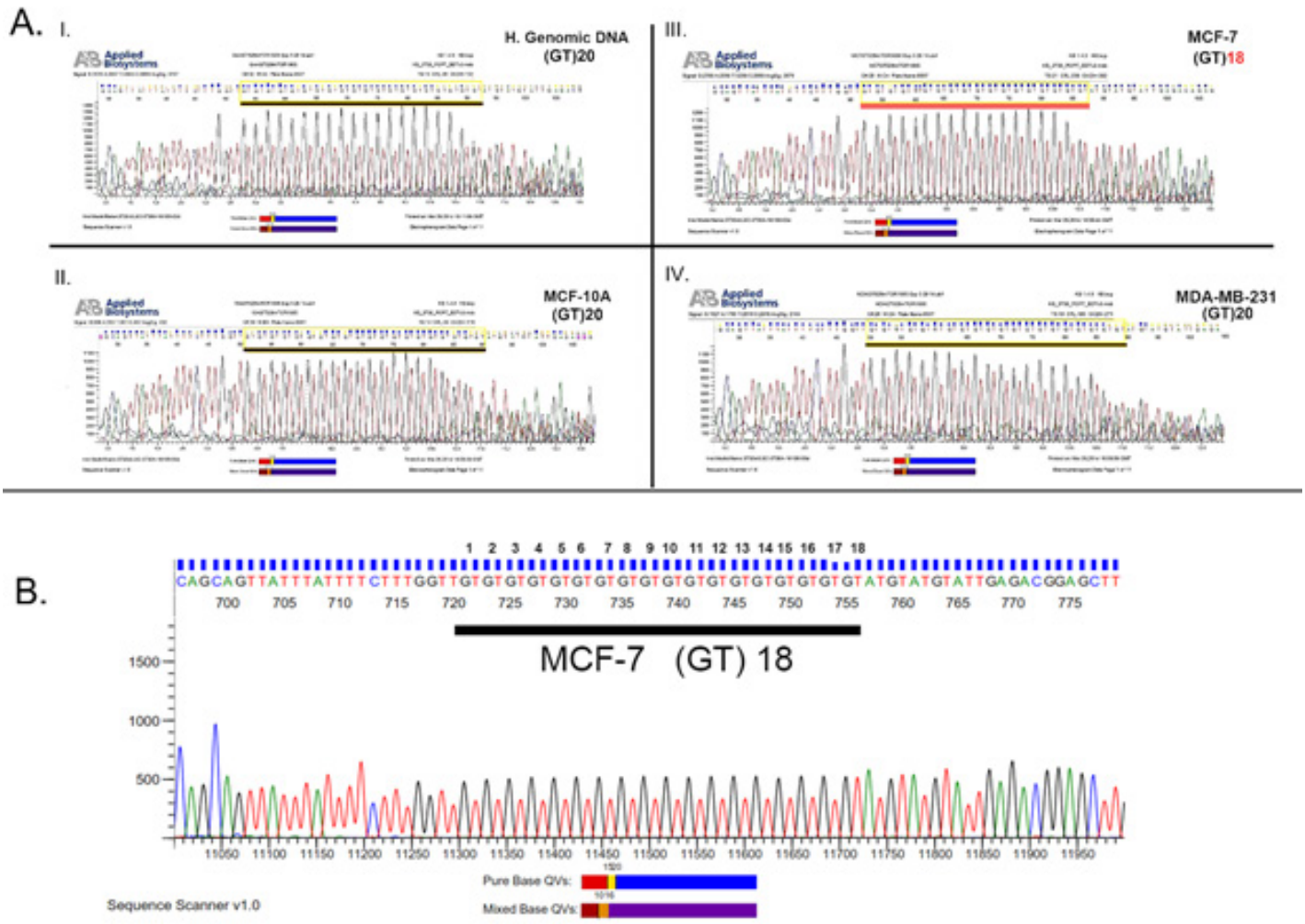

Figure 3. Length polymorphism of a dinucleotide repeats region in MTOR promoter. A. Chromatogram exhibits the $(GT)_n$ dinucleotide repeats region in the MTOR gene promoter obtained from I. normal human genomic DNA as well as genomic DNA obtained from II. MCF-10A, III. MCF-7 and IV. MDA-MB-231 breast cell lines as shown in the rectangles. **B.** Chromatogram exhibits the $(GT)_n$ dinucleotide repeats region in the *MTOR* gene promoter of genomic DNA obtained from MCF-7 cells shows the truncation of $(GT)_n$ region.

potentially involved in the regulation of the transcription of *MTOR* gene. For this purpose, multiple prediction tools of transcription factor binding sites including TRANSFAC (30), and RSAT (RSAT) (31) were used. Scanning of *MTOR* promoter region from human genomic DNA and from MCF-7 cells genomic DNA yielded a constellation of potential transcription factors, which possess possible binding sites in the DNA sequence. We compared the *MTOR* promoter sequences from human genomic DNA and the genomic DNA of the different breast cell lines using the NCBI nucleotide alignment tools for multiple sequences. Our findings revealed multiple sequence variations in the *MTOR* promoter of MCF-7 cells. As shown in figure 2, sequence variations of *MTOR* promoter in MCF-7 cells included single nucleotide polymorphism as well as single and tandem nucleotide repeats polymorphism. Comparison of the array of transcription factors potentially binding to MCF-7 *MTOR* promoter with those of human genomic *MTOR* promoter revealed some differences between the two sequences in the profile of potential transcription factors. Moreover, there are several putative transcription factors potentially binding to DNA of *MTOR* promoter in a close

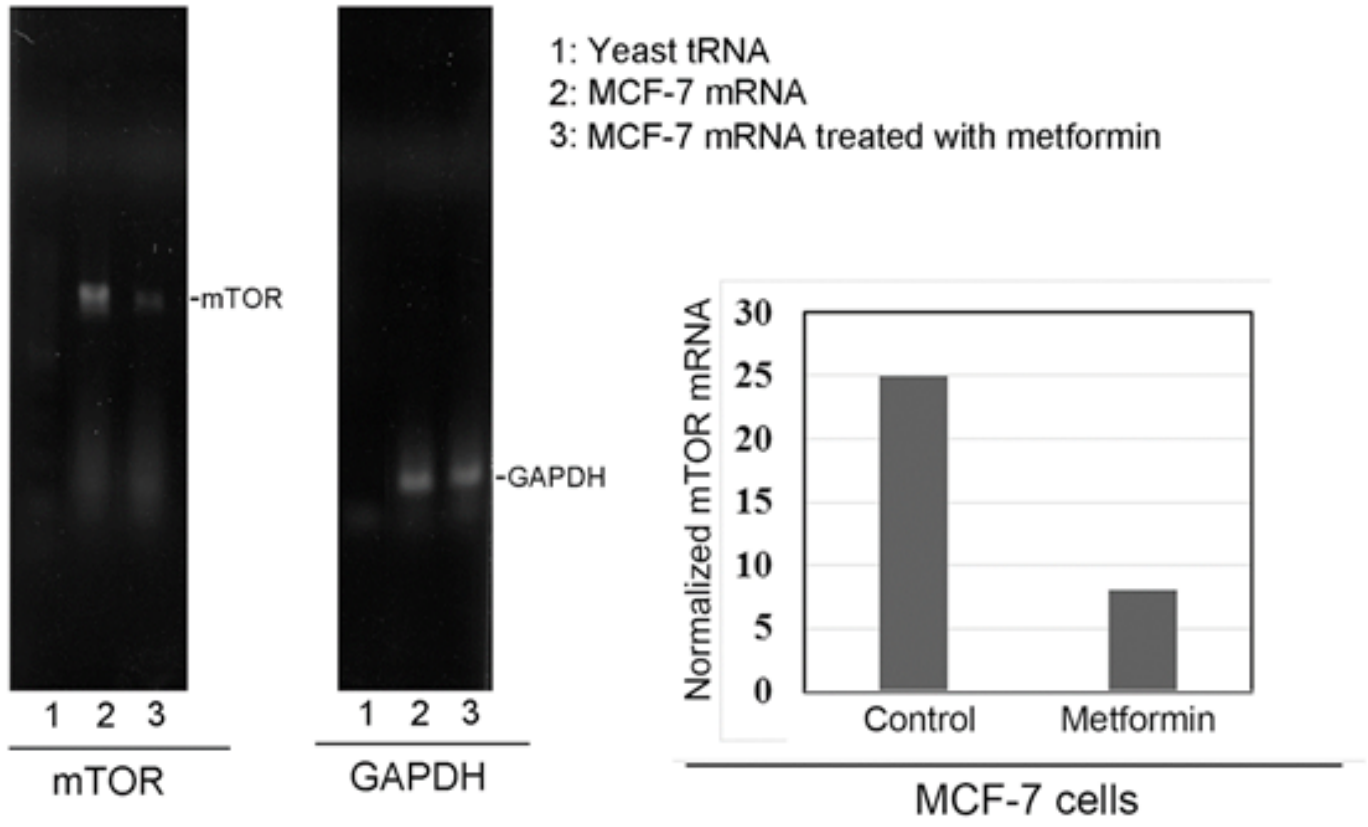

Figure 4. Metformin treatment was associated with significant decrease of mTOR mRNA level in MCF-7 breast cancer cells. Total RNA was isolated from untreated MCF-7 cells and cells treated with metformin 75mM for 8 hrs. The isolated RNA was then subjected to semi-quantitative RT-PCR analysis with primers specific for mTOR. The results were normalized to the level of GAPDH in each sample. The bar chart represents densitometry quantification of mTOR mRNA level in the study groups. Results of the effect of metformin treatment on total mTOR protein in MCF-7 cells was consistent with the results on mRNA level of mTOR in these cells following metformin treatment.

proximity to the sites of nucleotide variations in MCF-7 cells which could potentially affect *MTOR* gene expression in these cells.

MTOR promoter region in MCF-7 cells contains a truncated $(GT)_n$ dinucleotide repeat region.

Scanning of the *MTOR* promoter revealed a region of $(GT)_n$ repeats within the promoter of *MTOR* gene. Dinucleotide repeats in a gene usually exhibit length polymorphisms, which could potentially alter the gene transcription (22). As shown in the chromatogram results in Figure 3, the $(GT)_n$ region in the *MTOR* promoter of MCF-7 cells is truncated compared to the *MTOR* promoter obtained from the normal human genomic DNA, MCF-10A, or MDA-MB-231 cells. *MTOR* promoter sequence in MCF-7 cells contains $(GT)_{18}$ repeats compared to $(GT)_{20}$ in sequences of human genomic DNA. A duplicate sequencing of the *MTOR* promoter region from MCF-7 cells verified the shortening of $(GT)_n$ repeats. These findings suggest that the truncation in $(GT)_n$ dinucleotide repeats region MCF-7 cells is potentially involved in increasing transcription of *MTOR* gene in these cells.

Metformin treatment decreases mTOR mRNA and protein levels in MCF-7 cells.

Evidence shows that metformin inhibits transcription of some genes (). Our hypothesis was that metformin could be possibly involved in the transcriptional inhibition *MTOR* gene in MCF-7 cells. To test this hypothesis, we assessed the level of mTOR mRNA in MCF-7 cells treated with and without metformin treatment. As shown in figure 4, lane 3, metformin treatment was associated with significant

decrease in mRNA level of mTOR compared to the untreated MCF-7 cells figure 4, lane 2. These results suggest a role of metformin treatment in the inhibition of *MTOR* gene transcription in MCF-7 cells. Metformin treatment of MCF-7 cells was also associated with a significant decrease in the total level of mTOR protein in these cells as shown in figure 4. The role of metformin in inhibition of mTOR activity is well established (13), but these findings suggest a novel mechanism of action for metformin through the inhibition of *MTOR* gene expression. Such inhibition of *MTOR* gene expression could be possibly attributed to modulation of transcription factors which requires further investigation to elucidate it and potentially utilize it as a novel therapeutic approach.

Discussion

Our previous work revealed that total mTOR protein is higher in the breast cancer cells compared to the noncancerous cells. The elevated level of total mTOR protein correlated positively with the level of mTOR activity in MCF-7 cells. These findings suggest a role of total mTOR protein in promoting some of the phenotype of breast cancer cells, particularly, MCF-7 cells due to the apparent correlation between the total mTOR protein level and the level of mTOR activity in these cells. High protein level in a cell could be possibly attributed to decreased protein degradation, increased gene expression, or both. In another project, we have investigated the possibility of decreased mTOR protein degradation as an underlying cause of elevated mTOR protein level in breast cancer cells. In this study, we investigated the possibility of increased *MTOR* gene expression as a potential cause underlying the high mTOR protein level in breast cancer cells. Therefore, our next step was to evaluate the level of mTOR mRNA in different breast cell lines. Our results show that mTOR mRNA level is strikingly high in MCF-7 cells, as shown in Figure 1, lane 3, compared to the noncancerous MCF-10A breast cells Figure 1, lane 2. In contrast, the triple negative, MDA-MB-231, breast cancer cells did not show significant elevation of mTOR mRNA level compared to the noncancerous breast cells Figure 1, lane 4. These observations suggest a possible increase in the transcription of the *MTOR* gene in MCF-7 breast cancer cells.

Transcription of a gene is the first and the most common step in regulating the gene expression and gene promoters are important for the regulation of gene transcription (21). By comparing 1.9 kp DNA fragment of the *MTOR* promoter region obtained from normal human genomic DNA and from genomic DNA from different breast cancer cell lines, we identified a number of sequence variations. Our results showed sequence variation in the *MTOR* promoter of MCF-7 cells and potential variations in proteins binding adjacent to the sites of variations as shown in Figure 2. Among the sequence variations observed in the *MTOR* promoter of MCF-7 cells is the presence of shortening in a tandem dinucleotide $(GT)_n$ repeats region. Tandem dinucleotide repeats are, generally, prone to polymorphism (22), which might be linked to a number of clinical correlations in some genes (24-26). As shown in figure3A, panel III and figure 3.B, there is truncation of 4 nucleotides, $(GT)_{18}$ in MCF-7 cells compared to $(GT)_{20}$ in sequences of human genomic DNA as well as genomic DNA from MCF-10 and MDA-MB-231 breast cell lines as shown in Figure 3.A, panels I, II and IV, respectively. Elements of a gene promoter may serve as binding sites for various regulators of gene expression (21). Variations within gene promoters may lead to variations in the profile of binding proteins and consequently alteration the expression of the gene. These findings suggest that the truncation of this repeat region in MCF-7 cells might be involved in the upregulation of *MTOR* gene expression in these cells. Moreover, the $(GT)_n$ repeats, in particular, have the potential to form alternative DNA structures including Z- DNA, which acts as a binding site for proteins to control gene expression (22), which may lead to gene repression (28). Thence shortening of these dinucleotide repeats could be one of the underlying causes of the observed increase in *MTOR* gene transcription in MCF-7 cells, which requires further investigation for elucidation of its impact on the *MTOR* gene expression.

The findings of the present work also emphasized the results of previous research about the beneficial role of mTOR inhibition in breast cancer (9,11). Similar to direct mTOR inhibitors, metformin inhibits the phosphorylation and activation of mTOR and its downstream target P70-S6K (13). In corroboration with evidence from previous research, we found that the degree of inhibition of mTOR activity by metformin and rapamycin is comparable, but their effects on the cells proliferation, and viability are markedly different on MCF-7 cells in particular (data not shown). These findings emphasize the potential advantages of the use of metformin as an antineoplastic agent compared to allosteric and catalytic direct mTOR inhibitors (18), particularly, that these findings are further supported by mounting evidence of the antineoplastic effects of metformin from several epidemiological studies (32). Evidence from literature shows also that metformin

is capable of inhibiting the transcription of some genes. For instance, metformin inhibited mRNA expression of selenoprotein plasma1 (*SEPP1*) gene in hepatocytes *in vitro* and *in vivo* in a concentration- and time-dependent manner (33), which is consistent with previous reports of a strong reduction in the mRNA levels of selenophosphate synthetase 2 (*SPS2*) gene in primary hepatocyte cells after metformin treatment (34). Similarly, metformin treatment significantly inhibited transcription of the gluconeogenic genes; glucose-6-phosphatase (*G6pc*) and phosphoenolpyruvate carboxykinase 1 (*Pck1*) in the liver cells (35). The effects of metformin treatment on gene expression might involve modulation of certain transcription factors which alter gene expression through binding to the promoter region of the target genes. Metformin treatment has also been shown to reduce the level of some proteins such as the specificity protein (Sp) transcription factors in pancreatic cancer cells through the activation of proteasome-mediated degradation of these proteins, which is associated with inhibition of pancreatic tumor growth (36). Despite the well-known effect of metformin on the inhibition of mTOR phosphorylation and activation, the effect of metformin on the total mTOR protein level in breast cancer cells including its effect on *MTOR* gene expression is not known.

In conclusion, metformin treatment of MCF-7 cells was associated with marked decrease in the level of mTOR mRNA and protein. Although the reduction of total mTOR protein in MCF-7 cells induced by metformin could be possibly attributed to increased mTOR protein degradation, our data show that metformin treatment was associated also with decrease in mRNA level of mTOR. These findings suggest a role of metformin in inhibition of *MTOR* gene expression in MCF-7 cells. These findings provide a novel mechanism involving the mechanism of action of metformin in the ER+ MCF-7 breast cancer cells. These findings could be potentially utilized in improving the efficacy of breast cancer treatment modalities by including metformin as adjuvant chemotherapeutic agents in breast cancer treatment regimens.

Disclosure

No potential conflicts of interest.

Authors' contributions

Conception and design: M. Alalem, A. Ray, B.K. Ray; Development of methodology: M. Alalem, A. Ray, B.K. Ray Acquisition of data: M. Alalem, A. Ray, B.K. Ray; Analysis and interpretation of data: M. Alalem, A. Ray, B.K. Ray Writing, review, and/or revision of the manuscript: M. Alalem, A. Ray, B.K. Ray; Study supervision: B.K. Ray

Funding

This work was financially supported by the College of Veterinary Medicine Faculty Research Activity Grant and the Department of Veterinary Pathobiology for research assistantship (to M. Alalem).

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