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EXPERIMENTAL STUDY

Investigation of the effects of rapamycin on the mTOR pathway and apoptosis in metastatic and non-metastatic human breast cancer cell lines

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ABSTRACT

AIM: The aim of this study was to analyze the effects of rapamycin treatment on apoptosis via mTOR pathway in metastatic and non-metastatic human breast cancer cell lines by immunohistochemical and TUNEL analysis.

METHOD: MCF-7 and MDA-MB 231 cell lines were incubated under standard conditions forming Rapamycin and control groups. In immunohistochemical evaluation; mTOR pathway was evaluated with anti-IGF1, anti-PI3K, anti-pAKT1/2/3, anti-mTORC1, anti-mTORC2 and anti-ERK1 antibodies. The effect of apoptosis was also confirmed by TUNEL method.

RESULTS: In this study, activation of PI3K/AKT/mTOR and related molecular pathways in the MDA-MB 231 and MCF-7 breast cancer cell line was evaluated and it was observed that these pathways could play a key role in cancer development. Increased apoptotic cells were observed in mTORC1 inhibition by Rapamycin administration.

CONCLUSION: Targeting the mTOR pathway in breast cancer treatment may be a treatment option. In addition, the demonstration and confirmation of increased apoptosis in Rapamycin treated groups suggested that Rapamycin, an inhibitor of mTOR, is promising in the treatment of breast cancer *(Tab. 2, Fig. 3, Ref. 66)*.

Text in PDF *www.elis.sk.*

KEY WORDS: MDA-MB 231, MCF-7, breast cancer, mTOR, rapamycin.

Introduction

Breast cancer is the most common type of cancer in women worldwide (1). In 2018, around 600,000 women lost their lives due to breast cancer in the world (2). Invasive breast cancers express in about 75 % estrogen receptor (ER) and in about 30 % human epidermal growth factor receptor 2 (HER2) (3–5). More than 90 % of breast cancer-related deaths have been reported as complications caused by metastasis (6). Metastatic breast cancer lesions have been reported to develop in 30–60 % in the bone, 4–10 % in the brain, 15–32 % in the liver and 21–32 % in the lungs (7). Molecular mechanisms that determine which organs will be colonized by breast cancer are complex. However, breast cancer can be divided into four major clinical subtypes depending on gene expression profiles, estrogen (ER), progesterone (PR) or human epidermal growth factor receptor 2 (HER2) receptor and proliferation status (8). For the treatment of breast cancer, investigation of molecular mechanisms which effect the cell proliferation and survival are now at the forefront. In recent years, research has shown PI3K/AKT/mTOR and Ras/Raf/MEK/ERK pathways are frequently dysregulated in several cancers, including breast cancer (9, 10).

The PI3K phosphorylates phosphatidylinositol diphosphate (PIP2) to phosphatidylinositol triphosphate (PIP3) in order to promote the AKT activation (10). Akt activation leads to activation of mTORC1, which contributes to cell growth, proliferation and metabolism, but can also promote tumorigenesis (11–13). mTORC1 is activated by many metabolic molecular events such as growth factors, energy status, proinflammatory cytokines, oxygen levels, amino acids and canonical wingless type (Wnt) pathway. Growth factors such as insulin-like growth factor 1 (IGF1) which is one of the antibodies used in our study, receptor tyrosine kinases (RTKs) and PI3K/AKT pathway which is also subject to our study and Ras/Raf/Mek/Erk signal pathway are effective on mTORC1 (14, 15). Activation of MTORCl directly phosphorylates effectors such as ribosomal S6K1, leading to increased protein synthesis and cell survival. In addition, S6K1 activation has been reported to be involved in mTORC2 activation (16) and inhibits PI3K signaling, which further attenuates Akt activation (17).

mTOR is a serine/threonine protein kinase of the phosphoinositide 3-kinase (PI3K)-related kinase family, which plays a role in regulating cell growth, aging and metabolism (18). mTOR generally comprises two complexes, functionally and structurally different, called mTOR complex 1 (mTORC1) and mTOR complex

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2 (mTORC2) (18). mTOR is targeted by rapamycin, but mTORC1 and mTORC2 have different susceptibilities to rapamycin. Essentially, mTORC1 is directly inhibited by a complex formed by rapamycin and FKBP12 protein (19). But studies show that rapamycin did not inhibit mTORC2 (11, 20, 21). mTORC2 protein is thought to support cell viability by activating apoptotic protein BCL2 (B-Cell Lymphoma 2) by phosphorylating AKT and BAD proteins in the cytoplasm (22). The mTORC2 contains mTOR, but the most characteristic of this complex is that it phosphorylates the AKT protein (23). Interestingly, mTORC2 suppresses AKT signaling after stimulation by mTORC1 (24).

MAP kinases control cellular events for many complex short-term changes, such as embryogenesis, cell differentiation, cell proliferation and cell death, which are essential for homeostasis and acute hormonal responses (25). The MAPK signaling pathway consists of RAF, MEK and ERK proteins, which are essential for proliferation in many cancer cells as well as in normal cells (26). ERK1 and ERK2 are two identical proteins with a weight of 43 and 41 kDa and 83 % similarity (27, 28). Varied amounts of ERK1 and ERK2 were detected in almost all tissues, including differentiated tissues (25).

Purification of mTOR and the discovery of rapamycin as a physical target accelerated the studies in this field (29–31). mTOR inhibitors were originally developed as immunosuppressants, but have been approved for use in other cancers including renal cell carcinoma, neuroendocrine tumors of the pancreas, and subependymal giant cell astrocytomas as studies progress and new inhibitors emerge (32). The anti-cancer activity of rapamycin has been shown in several studies (33, 34).

In this study, we aimed to evaluate the effects of Rapamycin, which is an inhibitor of mTORC1, on the metastatic MDA-MB 231 and non-metastatic MCF-7 breast cancer cell lines with primary antibodies involved in PI3K, MAPK and apoptotic signal pathways using indirect immunohistochemistry and TUNEL methods.

Materials and methods

Cell culture and cell therapy

Metastatic (MDA-MB 231, 92020424, ECACC, Salisbury, UK) and non-metastatic (MCF-7, ACC115, DSMZ, Braunschweig, Germany) breast cancer cell lines were cultured in RPMI-1640 (F1213, Biochrom AG, Berlin, Germany) medium containing 10 % fetal bovine serum (FBS, S0113, Biochrom), 1 % L-glutamine (K0283, Biochrom) and 1 % Penicillin/Streptomycin (A2213, Biochrom). The cells were maintained at 37 °C in 5 % CO_2. Sterilized 12 mm diameter circular cover glasses (0.11152 million, Marienfeld Lab., Königshofen, Germany), were placed in 24-well plates. 500 µl FBS was added to each well of the plate for 30 min at room temperature. After aspiration of the FBS from the cells were plated in flasks and allowed to grow under routine conditions until about 70 % confluent. After growing the cells on 24 well-plate, the IC_{50} dose of Rapamycin (Rapamune, Sirolimus, Pfizer, Kent, UK) was determined as 1 µg/ml.

The experimental design was determined as the MDA-MB 231-control group (MDA-MB 231 metastatic breast cancer cells without exposure to any drug), MCF-7-control group (MCF-7 non-metastatic breast cancer cells without exposure to any drug); MDA-MB 231-Rapamycin group (MDA-MB 231 metastatic breast cancer cells treated with 1 ug/ml Rapamycin) and MCF-7-Rapamycin group (MCF-7 non-metastatic breast cancer cells treated with 1 ug/ml Rapamycin) evaluated the effect of 24-hour Rapamycin treatment.

Immunohistochemical evaluation

For the indirect immunohistochemical method cells were fixed in 4% paraformaldehyde in phosphate buffered saline solution (PBS) at 4 °C for 30 min and endogenous peroxidase activity was quenched by incubation with 3 % hydrogen peroxide (H_2O_2, K31355100, Merck, Darmstadt, Germany) for 10 min at room temperature. After washing with PBS cells were permeabilized with 0.1 % TritonX-100 (A4975, AppliChem, Darmstadt, Germany) in PBS at 4 °C for 15 min, and then cells were incubated with blocking serum (ready to use according to manufacturer's instruction, 85-9043, Invitrogen, Camarillo, CA, USA). Then anti-mTORC1 (Sc-27744, Santa Cruz Biotechnology, Heidelberg, Germany), anti-mTORC2 (Sc-130865, Santa Cruz Biotechnology, Heidelberg, Germany), anti-pAKT1/2/3 (Sc-135651, Santa Cruz Biotechnology, Heidelberg, Germany), anti-PI3K (Sc-1637, Santa Cruz Biotechnology, Heidelberg, Germany), anti-ERK (Sc-94, Santa Cruz Biotechnology, Heidelberg, Germany), anti-IGF1 (Sc-9013, Santa Cruz Biotechnology, Heidelberg, Germany), anti-caspase3 (Bs-0081R, Bios, MA, USA), anti-caspase8 (Bs-0052R, Bios, MA, USA), anti-caspase9 (Bs-0049R, Bios, MA,USA) and anti-APAF (Sc-65890, Santa Cruz Biotechnology, Heidelberg, Germany) all primary antibodies were diluted 1 : 100 in blocking serum for immunohistochemistry and incubated at 4 °C overnight. After washing with PBS, the secondary antibodies, biotinylated goat IgG anti rabbit/mouse IgG and peroxidase-conjugated streptavidin were incubated for 30 min for each step. Diaminobenzidine was applied to the cells as chromogen and for the background staining, Mayer's hematoxylin was used. Cells were covered with mounting medium, then viewed under light microscope (Olympus BX40). The distribution of immunohistochemical intensities of primary antibodies were scored as mild (+), moderate (++), strong (+++) and very strong (++++). After counting the percentage of positive staining cells, statistical significance was determined by assessment of differences using the ANOVA test. Significance was defined as $p < 0.05$.

Tunel evaluation

For the TUNEL method (In situ cell death detection kit, 11 684 817 910, Roche), cells were fixed in 4 % paraformaldehyde in phosphate buffered saline solution (PBS) at 4 °C for 30 min and endogenous peroxidase activity was quenched by incubation with 3 % hydrogen peroxide (H_2O_2, K31355100, Merck, Darmstadt, Germany) for 10 min at room temperature. After washing with PBS cells were permeabilized with 0.1 % TritonX-100 (A4975, AppliChem, Darmstadt, Germany) in PBS at 4 °C for 15 min, TUNEL reaction mixture was applied to the cells for 60 min at 37 °C in a humidified atmosphere in the dark. Then converter-POD

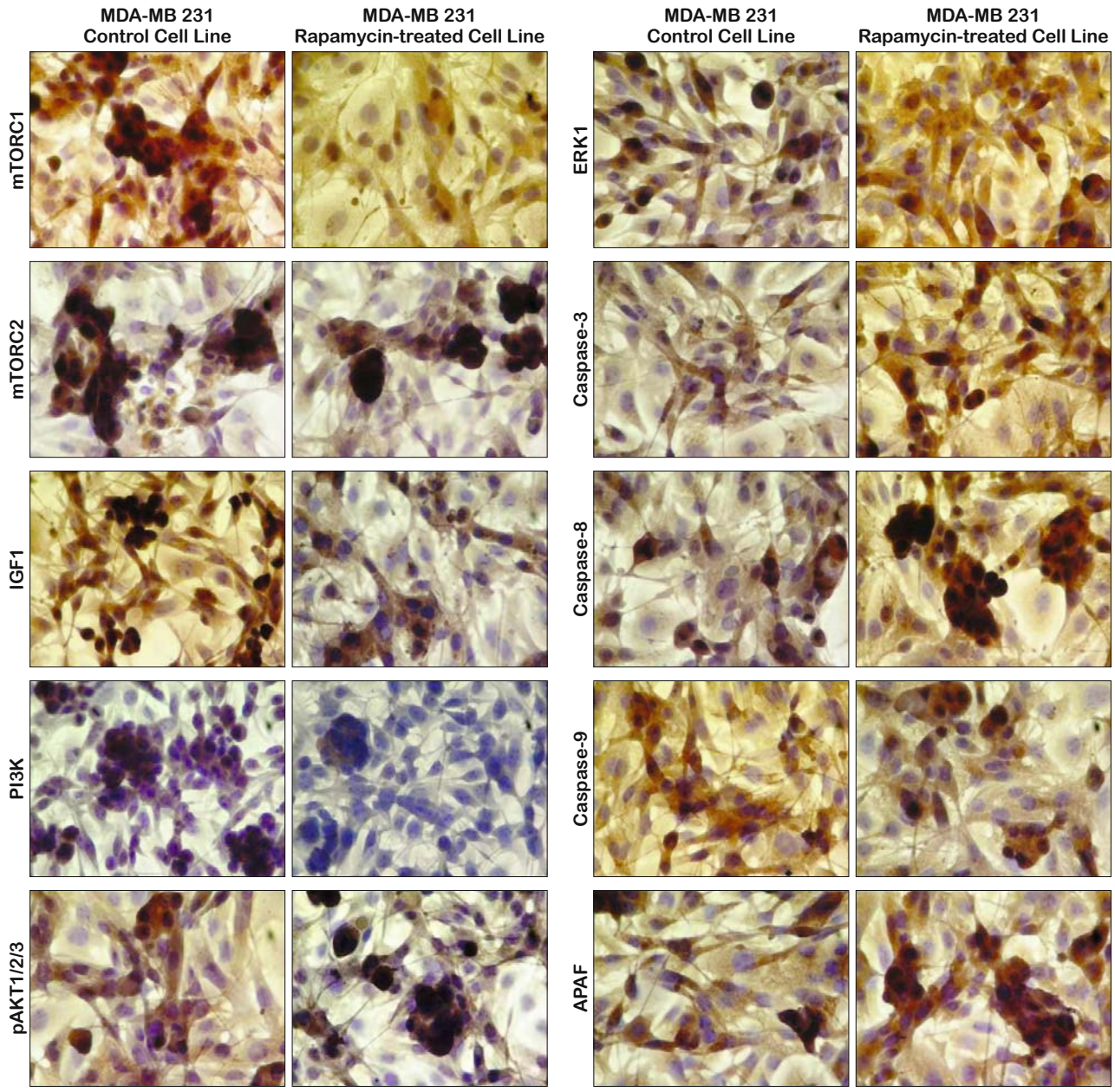

Fig. 1. Distribution of mTORC1, mTORC2, pAKT, PI3K, ERK, IGF, Caspase3, 8, 9 and APAF immunoreactivities of samples from non-treated control and Rapamycin-treated groups in MDA-MB 231 cell line. Original magnification: 400X.

solution was applied for 10 min at 37 °C. Diaminobenzidine was applied to the cells as chromogen and for the background staining, Mayer's hematoxylin was used. Cells were covered with mounting medium, then viewed under light microscope (Olympus BX40).

Results

According to the immunohistochemical evaluation, mTORC1, mTORC2 and IGF immunoreactivities were observed to be very strong, pAKT, PI3K and ERK immunoreactivities were strong in the control groups (metastatic MDA-MB 231 and non-metastatic MCF-7 cells without any treatment). In the control groups of both cell lines, the mTOR pathway molecules were determined to have similar immunoreactivities. Rapamycin treated MDA-MB 231 and MCF-7 cells showed high mTORC2, pAKT and ERK immunoreactivities while mTORC1 immunoreactivity statistically decreased in rapamycin treated groups compared with the control groups ($p < 0.001$). pAKT ($p < 0.01$) and ERK ($p < 0.05$) immunoreactivities were significantly increased in rapamycin treatment groups compared to control groups. mTORC2 immunoreactivity was similarly strong in both the control and the rapamycin treated groups. PI3K and IGF immunoreactivities were observed to be significantly decrease

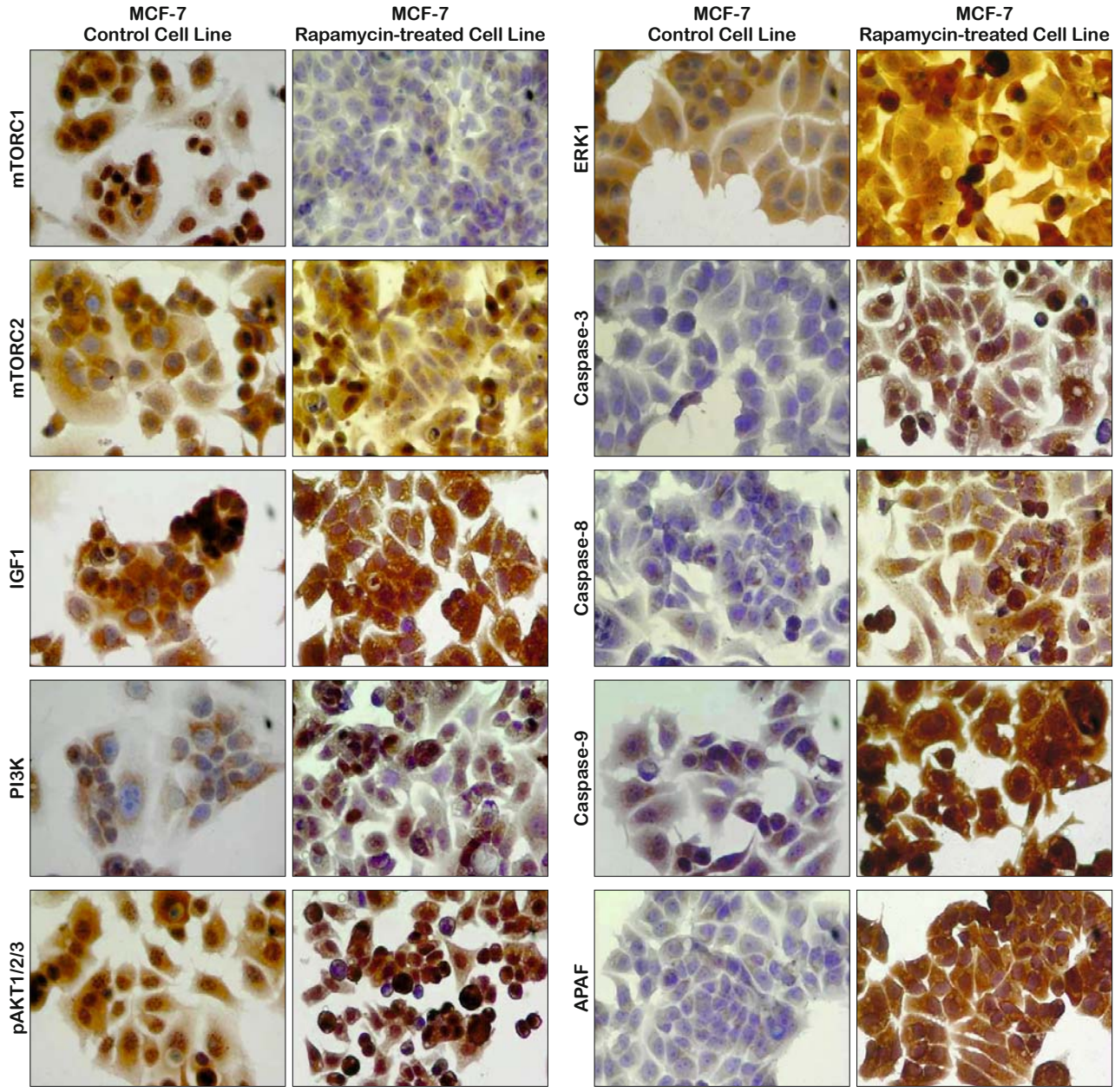

Fig. 2. Distribution of mTORC1, mTORC2, pAKT, PI3K, ERK, IGF, Caspase3, 8, 9 and APAF immunoreactivities of samples from non-treated control and Rapamycin-treated groups in MCF-7 cell line. Original magnification: 400X.

in rapamycin traeted MDA-MB 231 cells ($p<0.05$) while there was no difference in the rapamycin treated MCF-7 cells when compared to control groups. When examining the results, immunoreactivities were evaluated separately for each cell line (Figure 1 for MDA-MB 231 cell line, Figure 2 for MCF-7 cell line) (Tabs 1 and 2).

Caspase 3, 8, 9 and APAF immunoreactivities were moderate in the MDA-MB 231 control cells while caspase 3, 8 and APAF immunoreactivities were mild and caspase 9 immunoreactivity was moderate in MCF 7 control cells. In rapamycin treated groups caspase 3, 8, 9 and APAF immunoreactivities were observed as strong to very strong (Figs 1 and 2). When apoptotic markers were evaluated statistically, immunoreactivities of rapamycin treatment groups were found to be significantly increased ($p < 0.001$). (Figs 1 and 2). In concordance with the immunohistochemistry results there were much more TUNEL positive cells in rapamycin treated MDA-MB 231 and MCF-7 cell lines when compared with the non-treated MDA-MB 231 and MCF-7 cell lines ($p < 0.05$) (Fig. 3).

Discussion and conclusion

In this study we showed the activation of PI3K/AKT/mTOR and related molecular signal pathways in MCF-7 and MDA-MB

Tab. 1. Immunostaining intensities of mTORC1, mTORC2, IGF1, PI3K, pAKT1/2/3, ERK1 and Caspase-3, Caspase-8, Caspase-9 and APAF in MDA-MB 231 control and rapamycin-treated cell lines.

Antibody	MDA-MB 231 Control Cell Line	MDA-MB 231 Rapamycin-treated Cell Line
mTORC1	+++	++
mTORC2	++++	++++
IGF1	++++	+++
PI3K	+++	++
pAKT1/2/3	+++	++++
ERK	+++	++++
Caspase-3	++	+++
Caspase-8	++	++++
Caspase-9	++	++++
APAF	++	+++

Tab. 2. Immunostaining intensities of mTORC1, mTORC2, IGF1, PI3K, pAKT1/2/3, ERK1 and Caspase-3, Caspase-8, Caspase-9 and APAF in MCF-7 control and rapamycin-treated cell lines.

Antibody	MCF-7 Control Cell Line	MCF-7 Rapamycin-treated Cell Line
mTORC1	+++	++
mTORC2	++++	+++/++++
IGF1	++++	++++
PI3K	+++	+++
pAKT1/2/3	+++	++++
ERK	+++	++++
Caspase-3	++	++++
Caspase-8	++	+++
Caspase-9	++	++++
APAF	++	++++

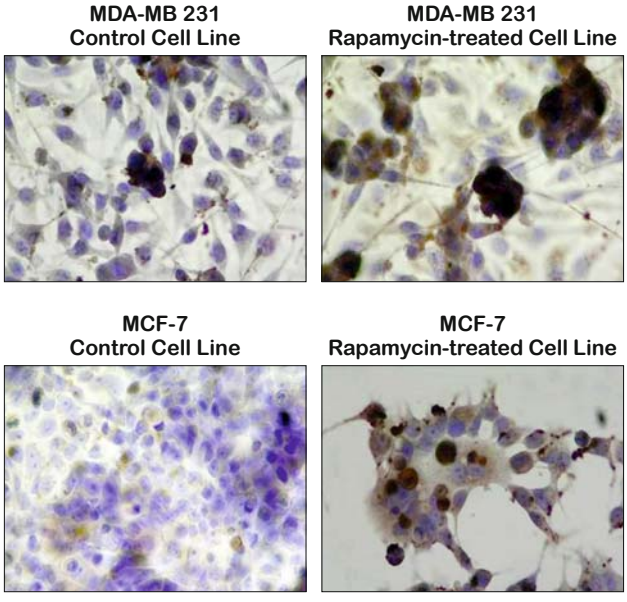

Fig. 3. TUNEL positive cells of samples from non-treated control and Rapamycin-treated groups in MDA-MB 231 and MCF-7 cell lines. Original magnification: 400X.

231 human breast cancer cell lines using indirect immunohistochemistry and TUNEL methods. It was observed that mTORC1 was inhibited by Rapamycin, while there was no change in mTORC2 immunoreactivity in both groups.

PI3K/AKT/mTOR and related Raf/MEK/ERK pathways are involved in the pathology of breast cancer. One of the major molecules in these pathways is mTOR. mTOR signals are often regulated in malignant tumors, including breast cancer, which emphasize the potential importance of mTOR-targeted therapy in cancer pathogenesis (35–38).

According to the immunohistochemical evaluation, mTORC1, mTORC2 and IGF immunoreactivities were observed to be very strong, pAKT, PI3K and ERK immunoreactivities were strong in the control group. Compared with the control group, as expected, mTORC1 immunoreactivity decreased in both rapamycin treated MDA-MB 231 and MCF-7 breast cancer cell lines. Also pAKT and ERK immunoreactivities increased in all rapamycin treated groups compared with the control. IGF1 and mTORC2 did not differ significantly. Since rapamycin is essentially an inhibitor of mTORC1, it was considered usual to have no changes in mTORC2. Increased pAKT and ERK immunoreactivities are expected because the increase in pAKT and ERK suppresses the transcription of mTORC1 (39, 40). Suppression of mTORC1 via pAKT or ERK was evaluated as a promising treatment of rapamycin in breast cancer.

The results of this study show that rapamycin has anti-cancer activity on metastatic MDA-MB 231 and non-metastatic MCF-7 breast cancer cells and this effect is mediated via the mTORC1 pathway, and rapamycin increases apoptosis in breast cancer cells.

mTOR is a downstream effector of PI3K and AKT. mTORC1 is the target of rapamycin and rapamycin analogues, such as everolimus, and leads to cell anabolic growth by promoting mRNA translocation and protein synthesis (41, 42) and also has roles in glucose metabolism and lipid synthesis. Its downstream substrate S6 kinase 1 can phosphorylate the activation function domain 1 of the ER, which is responsible for ligand-independent receptor activation (43, 44). Many mTORC1 functions are highly sensitive to rapamycin, whereas how FKBP12-rapamycin inhibits the activity of binding to mTORC1 is not fully known. It has been speculated that Rapamycin jeopardizes the structural integrity of mTORC1 and allostically reduces the specific activity of the kinase domain (23, 45, 46). Mutations and amplifications in genes encoding growth factors such as IGF1R (insulin-like growth factor 1 receptor) (47, 48), PIK3CA (49, 50), PDK1 (51), HER2 (52, 53) RAC-alpha serine/threonine protein kinase (AKT1) (54, 55) and fibroblast growth factor receptor 1 (FGFR1) (56) and loss of function or reduced expression of the genes encoding PTEN, 47 INPP4B (57, 58) and LKB1 (59) in breast cancer indicate the mTOR pathway.

Rapalog therapy may activate mTOR negative feedback on alternative pathways such as signaling pathways such as PI3K/AKT and MAPK/ERK (60–62). This observation highlights the need for alternative combinational therapeutic approaches for breast cancer treatment.

MAPK and PI3K/AKT pathways stimulate cell proliferation and increase cell survival through activation of transcription factors and cyclin D1 upregulation (63). PI3K is the main regulator of AKT activation and myogenesis (64). Combination of the rapamycin analogue everolimus and an ATP-competitive mTOR inhibitor BEZ235 showed synthetic lethality in several mTOR-addicted triple-negative breast cancer (TNBC) cell lines (65). Given the fundamental role of mTOR pathway signaling in the maintenance of ER positive breast cancer and its emerging role in resistance to endocrine therapy, strategic inhibition of pathway signaling must be considered in the treatment of ER+ breast cancer. This rationalizes the use of endocrine therapy and mTOR pathway inhibition in combination (49).

Abrogation of the negative feedback loop by mTORC1 inhibitors has only been demonstrated to influence PI3K/AKT signaling, whereas the impact of mTORC1 inhibition in other prosurvival pathways has not been addressed (60). This negative feedback loop has been directly related to the indolence of some types of cancers, suggesting that tumors with aberrant mTORC1 activation may in turn display reduced PI3K/AKT activity. Some researches have shown that the activation of mTORC1 regulates the expression of PDGFRs and that rapamycin treatment restores PDGFR levels and therefore PI3K signaling (66).

It was seen that Caspase 3, 8, 9 and APAF immunoreactivities were increased in rapamycin treated groups compared with the control groups. It was thought that both intrinsic and extrinsic pathways of caspase activation were involved in rapamycin induced apoptosis. In conclusion, these pathways may play an important role in cancer pathogenesis and new drug development for PI3K and mTORC2 inhibition is required.

In our study, activation of molecular pathways associated with PI3K/AKT/mTOR and ERK in metastatic MDA-MB 231 and non-metastatic MCF-7 human breast cancer cell lines was evaluated and it was observed that these pathways could play a key role in the cancer survival.

It was considered that mTORC1 inhibition was achieved with Rapamycin administration for 24 hours and apoptotic cells were increased in cancer groups and would be suitable for use in treatment. It was also thought that increased immunoreactivity of PI3K, AKT and ERK may be developed by negative feed back mechanisms and it may be important to develop drug protocols for inhibition of these pathways.

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