Original Article / Özgün Araştırma

DNA Methyltransferase Expression and Proliferation Status of Metastatic Breast Cancer Cell Line After Prolonged and Repeated Rapamycin and Melatonin Application

Uzun Süreli ve Tekrarlı Rapamisin ve Melatonin Uygulaması Sonrası Metastatik Meme Kanseri Hücre Hattında DNA Metiltransferaz Ekspresyon ve Proliferasyon Durumu

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Key words
Breast cancer, DNA methylation, Melatonin, Rapamycin

Anahtar kelimeler
Meme kanseri, DNA metilasyonu, Melatonin, Rapamisin

Received/Geliş Tarihi : 20.07.2015
Accepted/Kabul Tarihi : 21.07.2015

doi:10.4274/meandros.2117

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This study was presented as a poster in "1st Congress on stem cell and cell therapies with international participation, 20-23 March 2014, Kocaeli-Turkey".

Abstract

Objective: The aim of this study was to investigate the effects of Rapamycin and Melatonin and their combination on deoxyribonucleic acid (DNA) methylation and cell proliferation in a estrogen receptor (ER)-negative breast cancer cell line (4T1 cell line).

Materials and Methods: Four groups were designed with 4T1 cell line depending on drug combination (control, Rapamycin, Melatonin, Rapamycin + Melatonin) and their administration on different time periods (24, 48 and 72 hours). The drugs were administrated for 1, 2 and 3 times, respectively for these time periods. All samples were counted; immunostained (Ki67, DNA methyltransferase-1 (DNMT-1), DNA methyltransferase-3a (DNMT-3a) and p53) and real-time polymerase chain reaction (PCR) (DNMT-1 and DNMT-3a) was performed.

Results: The live/dead cell ratios were decreased in the Rapamycin and Rapamycin + Melatonin applied groups. Ki67 immunostaining showed that there was a decreased proliferation in the drug applied groups at 48th hours compared to the 24th hours. Also DNMT-1 expressions were decreased at 72th hour compared to that at 24th hour in all groups, especially in the Rapamycin administrated group. Adversely, DNMT-3a expression was increased at 72th hour compared to that at 24th hour in the groups, especially in the Rapamycin administrated group. Furthermore, an increased expression of p53 was seen in the drug given groups (highest in the Rapamycin applied group) when the time prolonged. Real-time RT-PCR analysis of DNMT-1 gene expression showed a decreased expression level in the Melatonin given group compared to the control group and an increased expression level was seen in the Rapamycin and Rapamycin + Melatonin administrated groups compared to the control group.

Conclusion: As a result, it was found that Rapamycin is more effective in metastatic breast cancer cells than Melatonin, both in the manner of cell viability and expressional changes of Ki67, DNMT-1, DNMT-3a and p53.

Özet

Amaç: Bu çalışmanın amacı; Rapamisin, Melatoninin ve bunların birlikte kullanımlarının, östrojen reseptörü (ER)-negatif meme kanseri hücre hattının (4T1 hücre hattı) deoksiribonükleikasit (DNA) metilasyonu ve hücre proliferasyonu üzerine olan etkilerini incelemekti.

Gereç ve Yöntemler: İlaç kombinasyonlarına (kontrol, Rapamisin, Melatonin, Rapamisin + Melatonin) ve bu ilaçların farklı zaman dilimlerinde (24, 48 ve 72 saat) uygulanmalarına bağlı olarak; 4T1 hücre hattı ile dört farklı grup oluşturuldu. İlaçlar, bu zaman dilimleri için sırasıyla; 1, 2 ve 3 defa uygulanmıştır. Tüm örnekler ile hücre sayımı, immun-boyama (Ki67, DNA metiltranferaz-1 (DNMT-1), DNA metiltransferaz-3a (DNMT-3a) ve p53 antikorları ile) ve Real-time PCR (polimeraz zincir reaksiyonu) (DNMT-1 ve DNMT-3a primerleri ile) yapıldı.

Bulgular: Rapamisin ve Rapamisin + Melatonin uygulanmış olan gruplarda canlı/ölü hücre oranı düşüş göstermiştir. İlaç uygulanan gruplarda 24. saate kıyasla 48. saatte, Ki67 immun-boyaması ile proliferasyonda düşüş gözlenmiştir. Ayrıca DNMT-1 ekspresyonları; 72. saatte, 24. saate kıyasla tüm gruplarda, özellikle rapamisin uygulanan grupta, düşüş göstermiştir. DNMT-3a ekspresyonu ise 72. saatte, 24. saate kıyasla tüm gruplarda, özellikle rapamisin uygulanan grupta, artış göstermiştir. İlaç verilen gruplarda (en çok rapamisin uygulanmış olan grupta), ilaç uygulaması arttıkça ve süre geçtikçe artan p53 ekspresyonu gözlendi. DNMT-1 gen ekspresyonunun Real-time RT-PCR sonuçlarına göre, DNMT-1 gen ekspresyonu Melatonin uygulanan grupta kontrol grubuna kıyasla düşme eğilimi gösterirken, rapamisin ve Rapamisin + Melatonin uygulanan gruplarda kontrol grubuna göre artmıştır. DNMT-3a gen ekspresyonunun Real-time RT-PCR sonuçlarına göre ise, DNMT-3a gen ekspresyonu, tüm ilaç uygulanan gruplarda kontrol grubuna kıyasla artmıştır.

Sonuç: Rapamisinin, metastatik meme kanseri hücrelerinde hem hücre canlılığı açısından hem de Ki67, DNMT-1, DNMT-3a ve p53'ün ekspresyonel değişimleri açısından Melatoninden daha etkili olduğu bulunmuştur.

Introduction

Breast cancer is a frequently seen cancer type in women and is a public health problem which has an increasing incidence and mortality. Clinically, breast cancer can be classified into different subtypes: one of these classifications depends on hormone receptor profiles of breast cancer cells, which are estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (Her2-neu/HER2). This classification type includes; triple positive, luminal A, luminal B, Her2 type and triple negative breast cancer (TNBC: ER (-), PR (-), HER2 (-)) (1). TNBC accounts for 10-15% of all breast cancers and TNBC patients have poor treatment outcomes compared to the other breast cancers' subtypes. The reason for this situation is that TNBC is more aggressive and does not have potential chemotherapeutic targets (2).

In mammals, cell proliferation is important for different processes, such as embryogenesis, growth and tumorogenesis (3). It is known that cancer cells have abnormal cell growth and proliferation pattern. Proliferation plays an important role in the treatment of cancer, as increased proliferation tendency of cancer cells causes poor prognosis (4). Disruption of deoxyribonucleic acid (DNA) methylation is the other hallmark of cancer. In general, DNA methylation is lower in tumor cells than in normal tissues, but in some part of the genome of cancer cells exhibits increased DNA methylation, such as CpG islands (5). The methylation is important for gene expression and the maintenance of genome integrity. In mammals, there are DNA methyltransferase (DNMTs) enzymes which establishes or maintains the methylation of DNA. Three enzymatically active mammalian DNMTs exist; DNMT-1, DNMT-3A and DNMT3B. Beside these, there is DNMT3L which is a regulatory protein lacking catalytic activity. DNMT-1 is reponsible for maintenance of methylation patterns during cell division, but DNMT3 is responsible for de novo methylation (6).

Rapamycin and Melatonin are the drugs used for different types of cancer treatment, including breast cancer. Rapamycin is isolated from Streptomyces hygroscopicus and is an agent that blocks the mammalian target of Rapamycin (mTOR) (serine-threonine kinases) pathway, which plays a key role in aggressive tumor growth, proliferation and survival (7). Melatonin (N-acetyl-5-methoxytryptamine), which is important for controlling biological rhythms, is an indolic hormone, mainly secreted by the pineal gland (8). It is shown that Melatonin plays a main role in tumor growth inhibition and motility decrement. Growth-inhibitory effects of Melatonin on breast cancer cells have been studied both in vivo and in vitro. It was found that the effects of Melatonin are more remarkable on ER-positive breast cancer cells, than in other types of cancers and the mechanisms of these effects are recently well known. It reduces the mitogenic response of cells and impairs estrogen

signaling pathway through both down-regulation of ER expression and inhibition of ER binding to the DNA (9). It has been shown that physiological concentrations of Melatonin (10^{-9} M) reduces the invasive capacity of ER-positive cancer cells; MCF-7 human breast cancer cells (10). Some studies have shown that Melatonin could stop the growth of ER-positive breast cancer cell line but not ER-negative breast cancer cells (11). Recently, it has been shown that Melatonin (1 mM) can reduce the cell viability of ER-negative breast cancer cells in vitro (12). 4T1 breast cancer cells are epithelial breast cancer cells in BALB/cfC3H mice and have similar tumor growth pattern and metastatic properties to human breast cancer. Also, they are a model for stage IV human breast cancer, resistant to 6-thioguanine (ATCC® CRL-2539™) and it is thought that they are TNBC cells (13).

The aim of this study was to investigate the effects of prolonged Rapamycin, Melatonin and their combined administration on DNA methyltransferase, cell proliferation and tumor suppressor markers expression in the 4T1 breast cancer cell line.

Materials and Methods

Cell line and Cell Culture

As a breast cancer cell line, the 4T1 cell line (ATCC® CRL-2539™) was used. 4T1 cell line was obtained from Prof. Dr. H. Nur Olgun (Dokuz Eylül University, Institute of Oncology) and after several pasages, cells at the 24th pasage were used for this study. 4T1 cells were grown according to the American Type Culture Collection (ATTC) recommendations. They were cultured in a complete medium, including DMEM (Lonza, Basel, Switzerland), 10% FBS (Gibco, Burlington, ON, Canada), 1% penicillin-streptomycin (Lonza, Basel, Switzerland) and 1% L-glutamine (Lonza, Basel, Switzerland), at 37 ºC in a humidified 5% CO_2 incubator. The experiment was performed in 4 groups with 3 different time periods (24 h, 48 h, 72 h) for each of them. And drug administration was done for 1, 2 and 3 times, respectively for these time periods.

- Group 1: Control group
- Group 2: Rapamycin (100 nM) (BioShop, Burlington, ON, Canada) applied group
- Group 3: Melatonin (1 nM) (Sigma, Munich, Germany) applied group
- Group 4: Melatonin (1 nM) + Rapamycin (100 nM) applied group

Main stocks of drugs were dissolved in ethanol (Sigma, St Louis, MO, USA) in the appropriate amount and then diluted with complete medium for the administration. The experiment was started with the same number of cells in all groups. The first drug administration time was considered as a zero time point. After 24 hours, group of 24-hour was analysed and second drug administration was done to 48-hour and 72-hour groups. At the 48 hours, group of 48 hour was analysed and third drug administration was done to 72-hour group.

Cell Counting

After 0.25% trypsin (Lonza, Basel, Switzerland) incubation, the cells in each group and time period were treated with trypan blue vital stain (Sigma, St Louis, MO, USA) to determine the ratio of live/dead cells and then counted with a haemocytometer.

Immunohistochemical Staining

For immunohistochemistry, antibodies of Ki67 (cell proliferation marker), DNMT-1, DNMT-3a (DNA methylation markers) and p53 (tumor supressor marker) were used. The slides were fixed with paraformaldehyde (Sigma, St Louis, MO, USA), treated with 3% hydrogen peroxide (Fluka, Buchs, Germany), solution with methanol (MERCK MILLIPORE, Darmstadt, Germany), and incubated with blocking solution (Invitrogen, Carlsbad, CA, USA) for 15 minutes at room temperature. Then, the slides were incubated with primary mouse monoclonal antibodies for Ki67 (Thermo scientific, SP6, Waltham, MA, USA), DNMT-1 (Bioss, Woburn, MA, USA), DNMT-3a (Bioss, Woburn, MA, USA) and p53 (Thermo scientific, Waltham, MA, USA) at 1:100 dilution for 2 hours at room temperature. This process was followed by a broad-spectrum secondary antibody (Invitrogen, Camarillo, CA, USA) administration for 1 hour, then, HRP-streptavidin reagent was applied (Invitrogen, Camarillo, CA, USA) for 30 minutes. After that, slides were stained with DAB (Diaminobenzidine, Invitrogen, Carlsbad, CA, USA). As a final step, the slides were counterstained with Mayer's hematoxylin (Sigma, St Louis, MO, USA). All images were taken at 200× magnification. Five areas were examined from each slide, especially demarcated areas with brown staining. The intensity of immunohistochemical staining was graded as follows:

(-) no immunostaining, (+) weak, (++) moderate and (+++) strong. (-) and, (+) stained cells were considered as negatively stained. (++) and (+++) stained cells were considered as positively stained. Positively and negatively stained cells were counted and their ratio was calculated.

Total Ribonucleic Acid Extraction and Real Time Polymerase Chain Reaction Analysis

Real-time polymerase chain reaction (real-time PCR) was performed to determine the messenger ribonucleic acid (mRNA) levels of DNMT-1 and DNMT-3a in 4T1 cells, treated with Rapamycin and Melatonin. Total ribonucleic acid (RNA) from 4T1 cells was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA USA) and purified with columns and reagents (Quiagen) in accordance with the manufacturer's instructions. Complementary deoxyribonucleic acid (cDNA) synthesis kit (Roche, Indianapolis, IN, USA), SYBR Green methodology and primers of DNMT-1 and DNMT-3a were used to perform a real-time quantitative PCR (qPCR) (qRoche Lightcycler 480 system) analysis. The housekeeping gene beta-actin was used as the internal control for gene expression normalization. The cycle threshold (Ct) indicates the number of cycles at which the fluorescent signal exceeds the threshold in qPCR. The mRNA levels were calculated using the comparative ΔCt method (target gene/reference gene). There was an inverse correlation between ΔCt value and gene expression level. The results were evaluated mathematically.

Results

Cell Counting

As a result of cell counting, it was found that live/dead cell ratios decreased in Rapamycin applied (group 2) and Rapamycin + Melatonin applied (group 4) groups as time progresses. The highest decrement in the live/dead cell ratio was seen in Rapamycin applied group. On the contrary, an increment in the live/death ratio was found in the control (group 1) and Melatonin applied (group 3) groups (Figure 1).

Immunohistochemical Staining

Immunohistochemical staining results were evaluated through rating the positive stained cells to negative stained cells. Ki67 expression was found to be decreased in the drug given groups and an increased expression of Ki67 protein was seen in the control group at 48 hours, compared to that at 24 hours. In addittion, Ki67 protein level was found to be decreased in the Rapamycin and Rapamycin + Melatonin applied groups compared to the control group at the 24 and 48 hours. Also Melatonin applied group had decreased Ki67 expression at 48 hours compared to the control group (Table 1a, 1b) (Figure 2). DNMT-1 expression was decreased in all groups at 72 hours compared to the 24 hours. The decrement in Rapamycin applied group was the highest. When the results were compared to the control group, it was seen that DNMT-1 expression

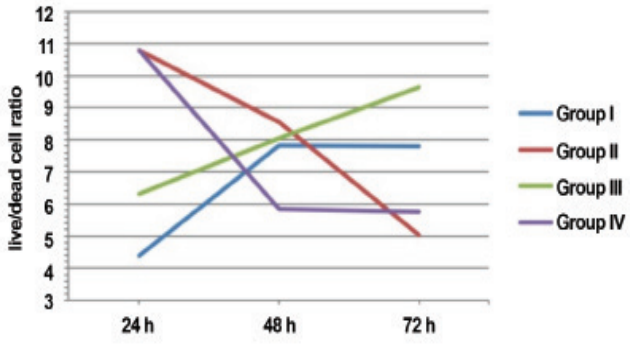

Figure 1. Live/dead cell ratio results: Group 2 (Rapamycin) and group 4 (Rapamycin + Melatonin) had a decrement, group 1 (control) and group 3 (Melatonin) had an increament in the live/dead cell ratio

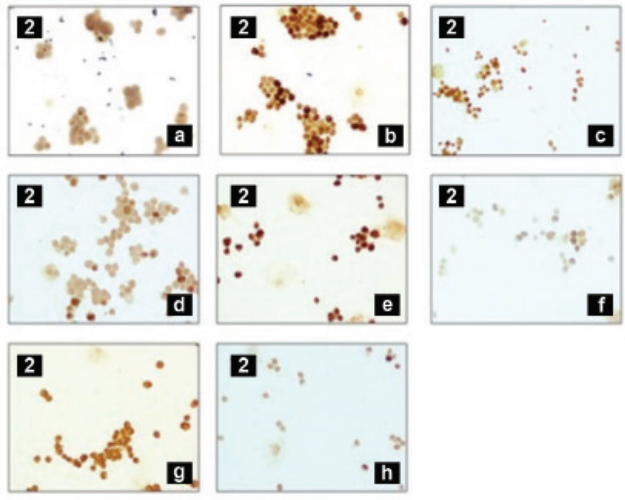

Figure 2. Immunostaining density of Ki67: a) Control group (24 hour) b) Control group (48 hour), c) Rapamycin applied group (24 hour) d) Rapamycin applied group (48 hour), e) Melatonin applied group (24 hour) f) Melatonin applied group (48 hour), g) Rapamycin and Melatonin applied group (24 hour), h) Rapamycin and Melatonin applied group (48 hour), (magnification: x200). Ki67 expression was decreased in drug treated groups and increased in the control group at the 48 hours, compared to the 24 hours

level was increased in the drug given groups at 24 hours, and at 72 hours, increased expression was seen in Rapamycin + Melatonin given group (Table 1 a-c) (Figure3). As a result of DNMT-3a immunohistochemical staining, an increased expression was found in the groups at 72 hours compared to the 24 hours, especially in the Rapamycin administrated group. However, when the results were compared to the control group, it was seen that there was a decreased expression in the rapamaycin and Rapamycin + Melatonin administrated groups at 24 hours. Besides, there was an increased expression in the Melatonin applied group at 24 hours compared to the control group. At the 48 hours, DNMT-3a expression level was increased in all drug given groups compared to the control group (Table 1 a-c) (Figure 4). An increasing expression of p53 in the drug given groups was found at 48 hours and 72 hours compared to the 24 hours, especially in the Rapamycin administred groups. And there was an increased expression level of p53 in the drug given groups at 48 and 72 hours compared to the control group (Table 1 a-c) (Figure 5).

Real-time Polymerase Chain Reaction

A. DNMT-1 Expression

DNMT-1 expression level was decreased in all groups as the time goes by. However, when the results were compared to the control group, it was

Table 1 a-c. Immunohistochemical staining results; ratio of positive stained cells (+) to negative stained cells (-) for four markers; a) at the 24 hour, b) at the 48 hour and c) at the 72 hour

Tablo 1. a. Groups of 24 hour (h), after 1 time drug administration

Groups (24 h)	Ki67 (+/-)	DNMT-1 (+/-)	DNMT-3a (+/-)	p53 (+/-)
Control	0.66	12.87	3.61	0
Rapamycin (100 nM)	0.46	20.54	1.31	0
Melatonin (1 nM)	2.41	18.62	4.5	0.71
Rapamycin (100 nM) + Melatonin (1 nM)	0.38	22.31	2.83	0

DNA: Deoxyribonucleic acid, DNMT-1: DNA methyltransferase-1, DNMT-3a: DNA methyltransferase-3a

Table 1b. Groups of 48 hour (h), after 2 times drug administration

Groups (48 h)	Ki67 (+/-)	DNMT-1 (+/-)	DNMT-3a (+/-)	p53 (+/-)
Control	0.71	0	1.64	1.28
Rapamycin (100 nM)	0.36	5.53	4.36	3.18
Melatonin (1 nM)	0.52	4.46	3.51	2.64
Rapamycin (100 nM) + Melatonin (1 nM)	0.37	1.97	13.84	2.76

DNA: Deoxyribonucleic acid, DNMT-1: DNA methyltransferase-1, DNMT-3a: DNA methyltransferase-3a

Table 1c. Groups of 72 hour (h), after 3 times drug administration

Groups (72 h)	Ki67 (+/-)	DNMT-1 (+/-)	DNMT-3a (+/-)	p53 (+/-)
Control	0	6.53	11.94	0
Rapamycin (100 nM)	4.14	6.49	32.82	7.48
Melatonin (1 nM)	11.84	5.51	4.85	3.42
Rapamycin (100 nM) + Melatonin (1 nM)	2.14	12.56	22.12	5.15

DNA: Deoxyribonucleic acid, DNMT-1: DNA methyltransferase-1, DNMT-3a: DNA methyltransferase-3a

found that DNMT-1 expression level was decreased in the Melatonin applied group at 24 and 72 hours. But DNMT-1 expression level was increased in the Rapamycin applied group at 24 and 48 hours compared to the control group. Also it was seen that DNMT-1 expression level was increased in the Rapamycin + Melatonin applied group in all time periods compared to the control group (Table 2a).

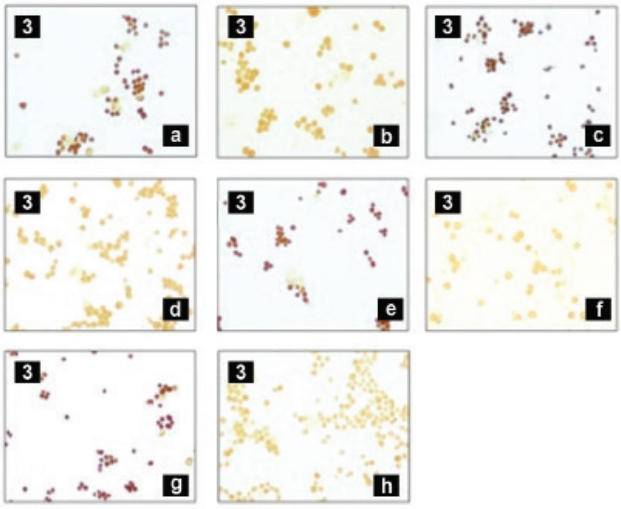

Figure 3. Immunostaining density of DNA methyltransferase-1 (DNMT-1): a) Control group (24 hour), b) Control group (72 hour), c) Rapamycin applied group (24 hour), d) Rapamycin applied group (72 hour), e) Melatonin applied group (24 hour), f) Melatonin applied group (72 hours), g) Rapamycin and Melatonin applied group (24 hour), h) Rapamycin and Melatonin applied group (72 hour) (magnification: x200). DNMT-1 expression was decreased at the 72 hours in all groups compared to the 24 hours

Table 2a, b. RT-PCR results; a) Relative expression ratio of DNMT-1 to reference gene and b) Relative expression ratio of DNMT-3a to reference gene

Tablo 2a. ΔCt value of DNMT-1

Groups	24 hour	48 hour	72 hour
Control	0.0150	0.0347	0.0443
Rapamycin (100 nM)	0.0128	0.0182	0.0830
Melatonin (1 nM)	0.0239	0.0340	0.0524
Rapamycin (100 nM) + Melatonin (1 nM)	0.0137	0.0239	0.0285

Table 2b. ΔCt value of DNMT-3a

Groups	24 hour	48 hour	72 hour
Control	0.2719	0.5262	1.3520
Rapamycin (100 nM)	0.1095	0.2072	0.9163
Melatonin (1 nM)	0.1500	0.2996	0.7393
Rapamycin (100 nM) + Melatonin (1 nM)	0.1200	0.3234	0.0741

B. DNMT-3a Expression

The expression level of DNMT-3a was decreased in all groups as the time goes by, but there was an increased expression in the Rapamycin + Melatonin applied group at 72 hours. However, when the results were compared to the control group, it was seen that

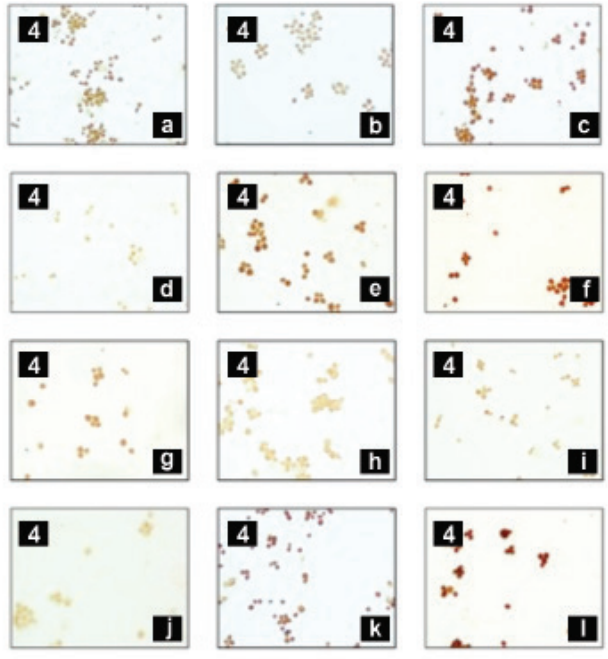

Figure 4. Immunostaining density of DNA methyltransferase-3a (DNMT-3a): a) Control group (24 hour), b) Control group (48 hour), c) Control group (72 hour), d) Rapamycin applied group (24 hour), e) Rapamycin applied group (48 hour), f) Rapamycin applied group (72 hour), g) Melatonin applied group (24 hour), h) Melatonin applied group (48 hour), i) Melatonin applied group (72 hour), j) Rapamycin and Melatonin applied group (24 hour), k) Rapamycin and Melatonin applied group (48 hour), l) Rapamycin and Melatonin applied group (72 hour) (magnification: x200). DNMT-3a expresison was increased at 72 hours in all groups compared to the 24 hours. Rapamycin and combined Rapamycin + Melatonin applied groups showed an increasing expression in each prolonged administration time. In the control and Melatonin applied groups a slight decreased expression at 48 hours compared to the 24 hours, and an increased expresssion at 72 hours compared to 48 hours was seen

DNMT-3a expression level was increased in all groups and in all time periods (Table 2b).

Discussion

Breast cancer is the most frequent cancer type in women and has an important rate of mortality (3). It has some routine prognostic markers; tumor size, grade, hormone receptor profile and genetic markers (14). Breast cancer is an important example of a hormone-dependent malignancy. The hormone estrogen has a role in the growth and differentiation of the normal mammary gland and the ductal branching. Despite this, the estrogen hormone has mammary carcinogenic effects as well (15). The cancer cells can acquire some properties which make them stronger than homeostasis mechanisms, such as unlimited proliferation, resistance to antiproliferative-apoptotic signals and escape from immune system (16). Besides, cancer cells undergo some changes in their genomic circuitry which allow them to survive. These changes may be activation of the protooncogenes or

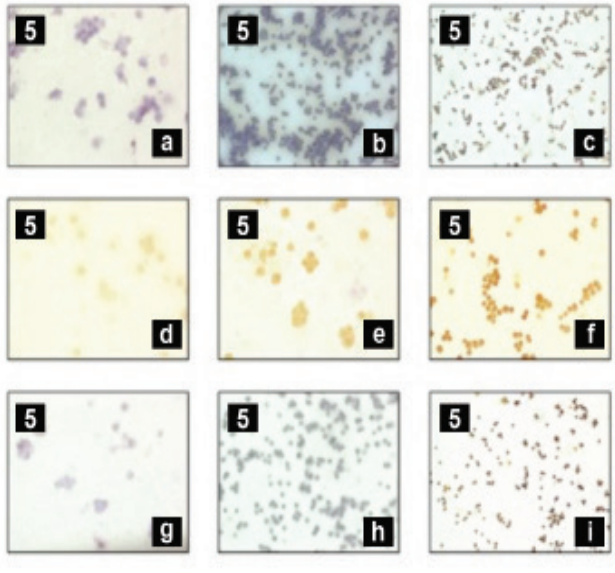

Figure 5. Immunostaining density of p53: a) Rapamycin applied group (24 hour), b) Rapamycin applied group (48 hour), c) Rapamycin applied group (72 hour), d) Melatonin applied group (24 hour), e) Melatonin applied group (48 hour), f) Melatonin applied group (72 hour), g) Rapamycin and Melatonin applied group (24 hour), h) Rapamycin and Melatonin applied group (48 hour), i) Rapamycin and Melatonin applied group (72 hour) (magnification: x200). In the drug given groups, an increasing density of p53 was found in each prolonged administration time

aberrant activation of tumor suppressor genes and pathways (17). Uncontrolled cell proliferation and disruption of methylation profiles of cells are the two hallmarks of the cancer (18,19). DNA methylation is an epigenetic event done by DNMTs. DNMT-1 has a role in maintenance of methylation profile, whereas DNMT-3A and DNMT3B carry out de novo methylation of genome (20). Changes in DNA methylation pattern of gene are associated with pathological conditions, such as cancer and autoimmune diseases (21). In this study, 4T1 (metastatic, 6-thioguanine-resistant) cell line was used and the effects of the drugs, Rapamycin and Melatonin, were investigated. The signaling pathways, which activate mTOR, a protein kinase that controls cell growth, proliferation and survival, were altered in different types of human cancers. It is already known that Rapamycin and its analogs block mTOR and, thereby, inhibit proliferation of cancer cells that leads to apoptosis (programmed cell death) (22). Rapamycin has effects on different types of cancer, such as rhabdomyosarcoma, neuroblastoma, glioblastoma, small-cell lung carcinoma, osteosarcoma, pancreatic carcinoma, renal cell carcinoma, Ewing's sarcoma, prostate cancer, and breast cancer (23). Melatonin is another drug that has an oncostatic role on several kinds of tumors, but especially on hormone-dependent mammary (24). It is already known that Melatonin has negative effects on ER-positive breast cancer (25,26). Besides, recently it has been shown that Melatonin has a role in reducing tumor growth and cell proliferation, and inhibition of angiogenesis of ER (-) breast cancer cells (12). In our study, it was found that there was an increment in cell viability in the control group as expected. Likewise, an increment in cell viability was found in the Melatonin applied group. In addition to this, in the Rapamycin applied group and Rapamycin + Melatonin applied group, there was a decrement in cell viability. The decrement in cell viabilty in the Rapamycin + Melatonin applied group was thought to be resulted from Rapamycin effects. These results indicate that Rapamycin has more adverse effect on cell viability than Melatonin. In accordance with these results, there are a lot of studies showing the adverse effects of Rapamycin even on triple-negative breast cancer cells when it is combined with some different agents as well (27,28).

Ki67 is a cell-cycle associated non-histone protein

required for cell proliferation and its level increases through mitosis. In this study, an increasing density of Ki67 in the control group at 48 hours showed us that there was an increment in proliferation in the cancer cells as expected. In contrast, there was a decrement in proliferation in all drugs applied groups in the time period of 48 hours. These results showed that drugs used in this study had adverse effects on cell proliferation of 4T1 breast cancer cells. Furthermore, these results are consistent with the cell counting results except for Melatonin applied group. In addition, DNMT-1 expression, which is one of the DNA methyltransferase and important for establishment of DNA methylation pattern, was decreased in all groups at 72 hours compared to 24 hours. Immunostaining of DNMT-3a showed an increased expression in all groups at 72 hours compared to 24 hours, which means that there were still ongoing methylations. Changes in methylation patterns of genomic DNA are generally assumed as an important factor of human cancers. As a result of these findings, it was thought that tumor suppressor genes may be activated after drug treatment due to the decrement in methylation and proliferation.

P53 is an important tumor suppressor gene and its activation can lead to either cell cycle arrest and DNA repair or apoptosis. As a result of immunostaining, increased level of p53 protein in the prolonged drug given groups was found especially in Rapamycin and combined Rapamycin-Melatonin applied groups. In a previous study, it was shown that DNMT-1 expression represses the p53 expression in the pancreas to ensure the lineage specification of pancreatic progenitor cells through prohibiting the p53-dependent apoptosis (29). In another study, it was shown that DNMT-3a directly interacts with p53 through the c-terminal domain of p53 in vivo and in vitro. This interaction causes the repression of p53 transcriptional activity which does not require DNA methyltransferase activity (30). In accordance with these studies, in this study, it was found that changes in p53 levels were compatible with the changes in DNMT-1 and DNMT-3a levels.

Conclusion

In this study, the effects of Rapamycin, Melatonin and their combinations on metastatic breast cancer cell line were investigated. The results obtained from cell counting and immunostaining showed that Rapamycin is more effective on cell proliferation and methylation of 4T1 cells. Besides, these effects became more prominent when the treatment period was prolonged. As a further study, searching for the expressional changes of other tumor suppressor genes' and downstream targets of p53 such as p21 is needed.

Acknowledgments

The authors thank Prof. Dr. H. Nur Olgun (Dokuz Eylül University, Institute of Oncology) for her kind support of providing the 4T1 cells.

Ethics Committee Approval: No ethical approval was needed, because it was an in vitro cell culture study, Informed Consent: Consent form was filled out by all participants, Concept: Kemal Ergin, Esra Gökmen, Design: Kemal Ergin, Esra Gökmen, Data Collection or Processing: Esra Gökmen, Analysis or Interpretation: Kemal Ergin, Esra Gökmen, Literature Search: Esra Gökmen, Writing: Esra Gökmen, Peer-review: Internal peer-reviewed, Conflict of Interest: No conflict of interest was declared by the authors. Financial Disclosure: The authors declared that this study has received no financial support.

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