MTUS1 TÜMÖR BASKILAYICI GEN VE ONUN ANA TRANSKRİPT VARYANTI ATIP3A'NIN U2OS VE MCF-7 HÜCRELERİNDE PAKLİTAKSEL KAYNAKLI NEGATİF REGÜLASYONU

Paclitaxel-induced Negative Regulation of MTUS1 Tumor Suppressor Gene and its Major Transcript Variant ATIP3a in U2OS and MCF-7 Cells

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ÖZET

Artan kanıtlar, MTUS1'in meme, yumurtalık, pankreas, kolon kanseri gibi çeşitli malignitelerde düşük seviyelerde eksprese edildiğini göstermiştir. Ayrıca, fonksiyonel çalışmalar, ATIP1 ve ATIP3a/b'nin, tümör baskılayıcı aktivitelere sahip olan MTUS1'in iki büyük splice varyantı olduğunu göstermektedir. ATIP3'ün bir mikrotübül ilişkili protein bölgesine sahip olduğu, mikrotübüllerle birlikte lokalize olduğu ve mikrotübül dinamiğinin düzenlenmesinde rol alarak uzamış mitoza yol açtığı rapor edilmiştir. Buna ek olarak, paklitaksel, çeşitli kanser tiplerinde güçlü etki gösteren ve mikrotübüllerin oluşumunu indükleyen ve bozulmasını engelleyen güçlü bir antineoplastik ajantır. Dolayısıyla, bu çalışmada tümör baskılayıcı MTUS1 ve MTUS1'in majör transkript varyantı olan ATIP3'ün paklitaksel ile indüklenmiş mikrotübül stabilizasyonu ve hücre ölümündeki rolününün araştırılması amaçlanmıştır. Paklitakselin aktif dozunu saptamak için, MCF-7 ve U2OS hücrelerinin hücre canlılığı, MTT hücre canlılığı testi kullanılarak belirlenmiştir. Ayrıca, toplam MTUS1 mRNA ve ATIP3 mRNA'nın gen ekspresyonundaki değişiklikler, real-time PCR metodolojisi kullanılarak belirlenmiştir. Sonuç olarak, MTUS1 ve ATIP3 ekspresyonun, MCF-7 hücrelerinde paklitaksel uygulaması sonrasında azaldığı tespit edilmiştir. MTUS1'in ekspresyon seviyeleri U2OS hücrelerinde de azalmıştır, ancak ATIP3'ün seviyesi artmıştır. Ancak, bu değişiklikler istatistiksel olarak önemsiz bulunmuştur. Sonuç olarak, paklitaksel U2OS ve MCF-7 kanser hücrelerinde MTUS1 tümör baskılayıcı geni ve MTUS1'in majör transkript varyantı olan ATIP3'ü negatif yönde düzenlemektedir. Bu sonuçlar MTUS1 ve protein ürünü olan ATIP3'ün mikrotübül dinamiğinin düzenlenmesinde rol oynayabileceğini göstermiştir.

Anahtar kelimeler: *ATIP; Cancer; İkili fonksiyon; MTUS1; MTSG1; Tümor Supresör*

ABSTRACT

Accumulating evidence proposes that MTUS1 was expressed at low levels in a variety of malignancies such as breast, ovarian, pancreas, colon cancers. Also, functional studies indicate that ATIP1 and ATIP3a/b are the two major splice variants of MTUS1 which possess tumor suppressor activities. ATIP3 was shown to bear a microtubule-associated domain and is localized in association with microtubules and interfere with the microtubule dynamics and leads to prolonged mitosis. In addition, paclitaxel is a talented antineoplastic agent which shows potent activity on various types of cancers and induces the assembly of microtubules and inhibits their disassembly. Accordingly, in this study, we aimed to examine the key role of tumor suppressor MTUS1 and its major transcript variant ATIP3 in paclitaxel-induced microtubule stabilization and cell death. To determine active dose of paclitaxel, cell viability of MCF-7 and U2OS cells was determined by using MTT cell viability assay. Also, gene expression changes of total MTUS1 and ATIP3 mRNAs were determined by using real-time PCR methodology. As a result, the expression of MTUS1 and ATIP3 was lowered in response to paclitaxel in MCF-7 cells. Expression levels of MTUS1 were also reduced in U2OS cells but ATIP3 was increased. However, these changes were statistically insignificant. In conclusion, paclitaxel is inducing negative regulation of MTUS1 tumor suppressor gene and its major transcript variant ATIP3a in U2OS and MCF-7 cells and they might be involved in the regulation of microtubule dynamics

Key words: *ATIP; Cancer; Dual functions; MTUS1; MTSG1; Tumor Suppressor*

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1. INTRODUCTION

Cancer is deadly major health situation which is estimated to be responsible for the 14.1 million new cases and 8.2 million annual deaths (1). It is clear that alterations in the proto-oncogenic and tumor suppressor signaling are the important hallmarks of cancers (2, 3). Particularly, the loss of tumor suppressor gene function is a common occasion in almost all cancers (2, 3). P53 is one of the well-known example in which the mutated p53 having lost its function to induce cellular apoptosis in harsh conditions (4). Moreover, MTUS1 is recently annotated as a novel tumor suppressor gene (5). It is first identified and characterized in pancreas cancer tumors and cells by Seibold et al (4). MTUS1 gene has been shown to be localized to 8p21.3-22 chromosomal region and approximately 150 kb in length (4). According to current knowledge, there are six different splice variants of this gene and five of them are known to produce five protein isoforms; ATIP1, ATIP2, ATIP3a, ATIP3b and ATIP4, called as angiotensin II receptor-interacting proteins (ATIPs) (6). ATIP1 is the primary identified member of this protein family and shown to be interacted with the C-terminus domain of AT2 receptor (7). Moreover, ATIP1, ATIP3a and ATIP3b variants were shown to have well tissue distribution and other members either poorly expressed or specifically expressed in some tissues (5). In addition, both ATIP3a/b were reported to be major protein isoform and ATIP1 was prominently expressed in brain tissues compared to other isoforms(5).

Furthermore, accumulating body of evidence suggests that MTUS1 was highly downregulated in a several types of malignancies such as colon, ovarian, breast, and pancreas cancers (8). Particularly, functional studies suggest that ATIP1 and ATIP3a/b have tumor suppressor activities. In addition, MTUS1 were primarily shown to be transiently stimulated in the course of initiation of quiescence and differentiation of cells (4). Furthermore, it was reported that ATIP3 has a microtubule-associated domain and is localized in association with microtubules (9, 10). Particularly, by co-localizing with microtubules, ATIP3 was shown to interfere with the microtubule dynamics and leads to prolonged mitosis (9, 10). In the confocal and fluorescence microcopy analyzes, it was shown that ATIP3 is localized in microtubule skeleton and microtubule organization centers with alpha-tubulin in cells expressing low ATIP3 (9, 10). It was also shown that elevated expression of ATIP3 triggers the formation of microtubule bundles (9, 10). This shows that ATIP3 can control the microtubules dynamic by triggering microtubule stabilization

Moreover, paclitaxel is a promising antineoplastic agent which shows potent activity on wide range of cancers (11). Paclitaxel induce the assembly of microtubules and inhibits their disassembly (11). Interestingly, assembled microtubules in the presence of paclitaxel are stable and dysfunctional and lead to cell death by disrupting the regulation of microtubule dynamics (11). Accordingly, in this study, main aim was to investigate the role of tumor suppressor MTUS1 and its major transcript variant ATIP3a in paclitaxel-induced microtubule stabilization and cell death

2. MATERIALS AND METHODS

2.1. Cell culture

U2OS and MCF-7 cancer cell line (American Type Culture Collection, VA, USA) were included in this study. Dulbecco's modified eagle's essential medium (DMEM) (GIBCO, NY, USA) supplemented with the 10% heat-inactivated fetal bovine serum (FBS) (Sigma-Aldrich, MO, USA) and penicillin (100 units/ml)/streptomycin (100 µg/ml) solution (Sigma-Aldrich, MO, USA) was used to propagate cells.

2.2. Determination of cell viability by MTT assay

Viability of cancer cells after Paclitaxel treatments were determined by using MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) cell viability assay and active doses were determined. Briefly, U2OS and MCF-7 cancer cells were treated different doses of Paclitaxel (0.03125, 0.0625, 0.125, 0.25 and 0,5 µM) (Catalog number: P3456) (Thermo Fisher Scientific Inc., Wilmington, USA) at 37ºC with 5% CO2 for 24h. After 24 hours of incubation with Paclitaxel, cells were incubated with the 1 mg/ml MTT solution for 1 hour and subsequently MTT was discarded. Plates were air-dried and purple formazan particles were dissolved using Dimethyl Sulfoxide (DMSO) (Sigma-Aldrich, MO, USA).

2.3. RNA isolation and cDNA synthesis

RNA was obtained from cultured cells using GeneJET RNA Purification Kit (Thermo Fisher Scientific Inc., Wilmington, USA) according to the manufacturer's recommended procedures. Quality and concentrations of RNA samples were determined by using NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, USA). For cDNA synthesis, RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific Inc., Wilmington, USA) were used. For the preparations of cDNA samples instructions of the supplier were followed and prepared samples were preserved at -80ºC in equal aliquots.

2.4. RT-qPCR experiments

Quantitative PCR (RT-qPCR) experiments were performed using Luminaris Color HiGreen qPCR Master Mix (Thermo Fisher Scientific Inc., Wilmington, USA) and the reactions were held in Rotor-Gene Q instrument (QIAGEN Sample & Assay Technologies, Germany). All experiments were studied in triplicate. Breifly, for the real-time PCR, following PCR mix were prepared for each reaction; 5 µL 2X Master Mix, 0.3 µL 10 µM Forward Primer, 0.3 µL 10 µM Reverse Primer, 1 µL cDNA and , nuclease-free water up to 10 µL. Reactions were subjected to following thermal cycling conditions; 10 min initial denaturation at 95 °C repeating 1 cycle, 15 s denaturation at 95 °C, 30 s annealing at 60 °C and 30 s Extension at 72 °C repeating 40 cycles. Also a melting curve analysis performed at the end of the reaction between 60 and 95 °C.

2.5. Analysis of data and statistical comparisons

GAPDH gene was used as internal reference control for all expression experiments. For the calculation of relative expression in qPCR experiments, 2−ΔCt (ΔCt = Target gene − Reference gene) formula was used. Also, a fold change analysis performed using the 2−ΔΔCt (ΔCt = Target gene − Reference gene) formula. Statistical analysis of the resulting data was achieved by using GraphPad Prism (v6.02, GraphPad Software, CA, USA) and SPSS (version 20, IBM, NY, USA) programs and Wilcoxon test applied. All statistical analysis was two-tailed, and $p < 0.05$ were accepted as significant.

3. RESULTS

3.1. Anti-proliferative activity of paclitaxel in U2OS and MCF-7 cells

To assess the anti-proliferative activity of paclitaxel in U2OS osteosarcoma and MCF-7 breast cancer cells, MTT cell viability assay was used. As a result, significant anti-proliferative activity of paclitaxel was observed with the increasing levels of treatment and 0.5 µM of doses of paclitaxel used was determined in both cell lines as active dose (Figure 1).

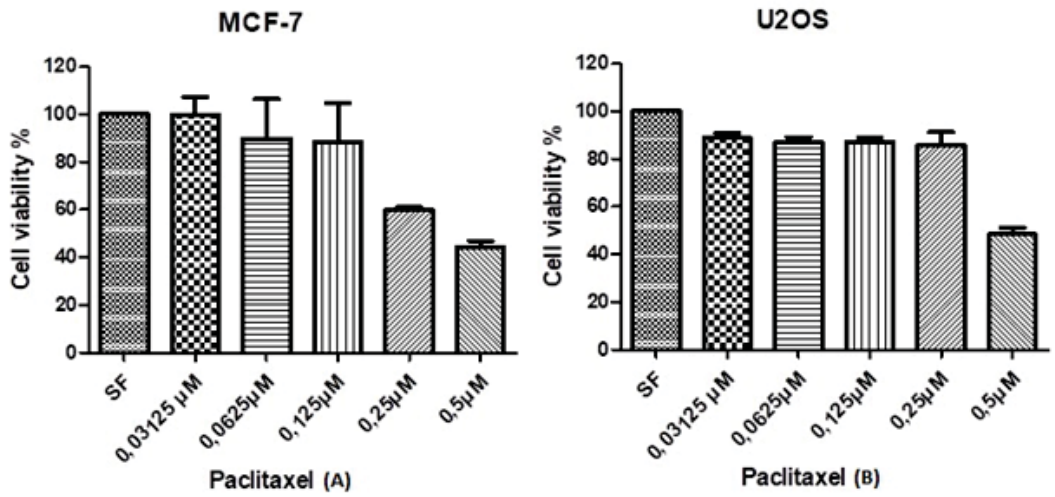

Figure 1. Anti-proliferative activity of Paclitaxel on A) MCF-7 breast cancer cells B) U2OS osteosarcoma cells.

3.2. Gene expression analysis of total MTUS1 expression levels and ATIP3a isoform

To ascertain the effect of paclitaxel on the expression of MTUS1 tumor suppressor gene and its major transcript variant ATIP3a, RT-qPCR technique was utilized. Accordingly, quantitative expression levels of total MTUS1 mRNA and ATIP3 mRNA were found to be down-regulated as result of paclitaxel treatments in MCF-7 breast cancer cells (Figure 2).

Moreover, a fold-change analysis was also performed and significant downregulation of MTUS1 and ATIP3a was observed in MCF-7 breast cancer cells (Table 1). Furthermore, while the quantitative expression levels of total MTUS1 mRNA was shown to be down-regulated as result of paclitaxel treatments in U2OS osteosarcoma cells, ATIP3a expression levels was upregulated (Figure 3).

Fold-change analysis in U2OS cell lines also confirmed these results (Table 1). Statistical analysis revealed no significant alteration in gene expressions ($p>0.05$).

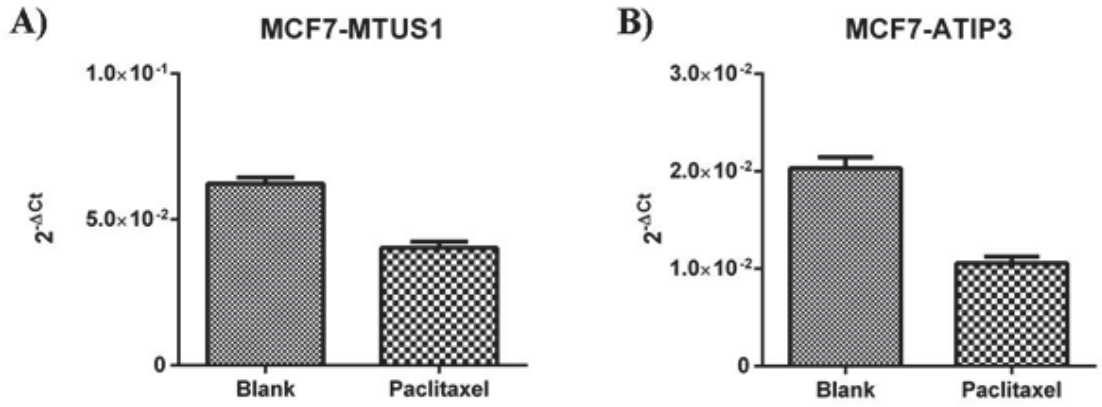

Figure 2. Differential expression of MTUS1 and its major transcript variant ATIP3a in MCF-7 breast cancer cells.

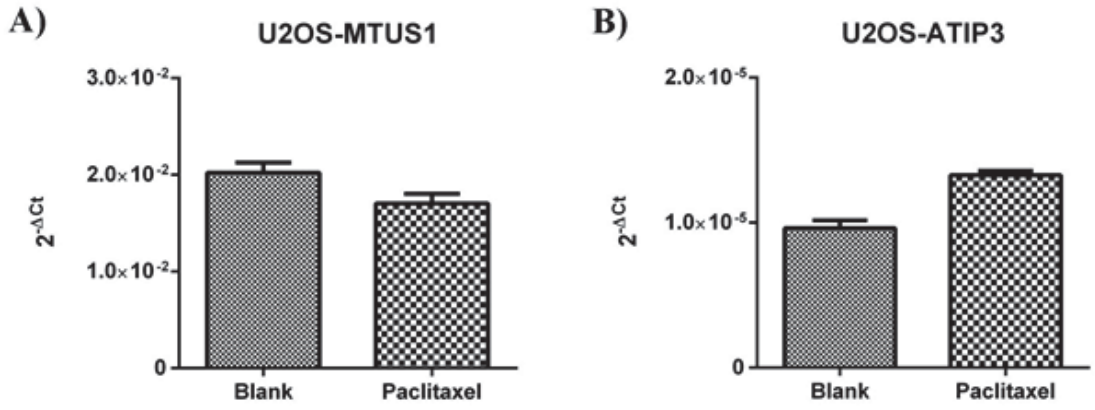

Figure 3. Differential expression of MTUS1 and its major transcript variant ATIP3a in U2OS osteosarcoma cells.

Table 1. Fold-change differences of total MTUS1 mRNA and ATIP3a transcript variant in MCF-7 breast cancer and U2OS osteosarcoma cells after paclitaxel treatments.

	MCF-7		U2OS	
	Fold change	p value	Fold change	p value
MTUS1	0.650670928	p>0.05	0.829319546	p>0.05
ATIP3a	0.52123288	p>0.05	1.328685814	p>0.05

4. DISCUSSION

Microtubules are dynamic structures which steadily transit between polymerization and depolymerization phases at their plus ends (12). This condition is actually well-known as microtubule dynamic instability which enables cells to respond various intracellular and extracellular stimuli (12). Defects in microtubule dynamic instability were shown to alter the regulation of mitotic spindle formation, chromosome segregation, migration, as well as transport of proteins and organelles within the cell (9, 12). Therefore, it leads to the formation and progression of cancer (9, 12).

The proteins that regulate microtubule dynamic instability are so called Microtubule-associated proteins (MAPs) (12). ATIP3, which is encoded by MTUS1 gene, is a recently identified MAP (12). Moreover, accumulating body of evidence suggests that MTUS1 and its transcription variants is heavily involved in the initiation, progression and metastasis of human cancers (6, 9). Studies also indicated that ATIP1 and ATIP3 is the major splice variants of MTUS1 gene and have significant tumor suppressor function in different types of human cancers (6, 9). In particular, similar to ATIP1 and ATIP3 was shown to interfere with the activation of extracellular signal-regulated kinase (ERK) signal transduction (13, 14). Particularly, recombinant overexpression of ATIP3 was shown to obstruct proliferation and migration of SACC-LM cells through downregulating the activities of ERK, and mesenchymal markers of Slug and Vimentin (14). In addition, as a microtubule-associated protein, ATIP3 was shown interact with microtubules (12, 15). Increased expression of ATIP3 has been shown to trigger the formation of microtubule bundles, thus in turn regulating microtubule dynamics (12, 15). It has also been reported that ATIP3 sediments with stabilized microtubules (12, 15). It has been also shown that microtubule dynamics are altered in cells stably expressing ATIP3 and microtubule bundles decrease nocodazole susceptibility (15). In addition, ATIP3 has shown to interact with EB1 (End-binding protein 1) and increased expression of ATIP3 suppresses EB1 accumulation at microtubule plus ends independent of microtubules (12). More recently, it was shown that ATIP3 interferes with the proliferation, migration and metastasis of breast cancer cells through altering microtubule dynamics (15). In short, these findings strongly indicate that ATIP3 interacts with microtubules and EB1 protein, regulating microtubule dynamic instability and delaying the progression of mitosis and limiting cellular migration.

Paclitaxel is a taxane drug which is routinely used to fight against various cancer types in cancer therapy. Mechanism of action of paclitaxel involves the stabilization of microtubules. Accordingly, in this particular study, it was tried to reveal the role of tumor suppressor MTUS1 and its major transcript variant ATIP3a in paclitaxel-induced microtubule stabilization and cell death. Surprisingly, expression levels of both MTUS1 and its major splice variant ATIP3 was found to be diminished in paclitaxel treated breast and osteosarcoma cancer cells. Strongly suggesting that paclitaxel is inducing negative regulation of MTUS1 tumor suppressor gene and its major transcript variant ATIP3a in U2OS and MCF-7 cells. These findings also support the notion that MTUS1 and ATIP3 splice variant might be involved in the regulation of microtubule dynamics.

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