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The effect of DNA methyltransferase 3A suppression in progression of the resistance phenotype in breast cancer cells

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Introduction. Rearrangement of molecular pathways and activation of bypass signaling determine the progression of tumor cell resistance to various drugs. Study of the common features of resistant formation mechanisms is essential for breast and other cancer beneficial treatments.
Materials and methods. The present work was performed on estrogen receptor α ERα-positive (ERα – estrogen receptor α) MCF-7 breast cancer cells, established sublines resistant to the mTOR inhibitor rapamycin or antiestrogen tamoxifen, and ERα-negative MDA-MB-231 breast cancer cells. Methods used include MTT test, transient transfection, immunoblotting, real-time polymerase chain reaction and methylation analysis by bisulfite pyrosequencing.
Results. We have shown that the resistance of breast cancer cells to targeted and hormonal drugs is associated with the suppression of DNA methyltransferase 3A (DNMT3A) and respective changes in DNA methylation; DNMT3A knockdown results in the partial resistance to both drugs demonstrating the pivotal role of DNMT3A suppression in the progression of cell resistance.
Conclusion. Totally, the results obtained highlight the possible mechanism of tumor cell resistance to targeting/hormonal drugs based on the deregulation of DNMTs expression and demonstrate direct connection between DNMT3A suppression and resistance progression.

Keywords: rapamycin, tamoxifen, drug resistance, MCF-7 cells, protein kinase AKT, DNA methyltransferase, LINE repeats

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Феномен подавления ДНК-метилтрансферазы 3А при формировании резистентного фенотипа в клетках рака молочной железы

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Введение. Переключение сигнальных путей и активация параллельных сигнальных каскадов относятся к ключевым факторам, определяющим развитие резистентности опухолевых клеток, и изучение механизмов подобной реаранжировки является одной из актуальных задач современной онкологии.

Материалы и методы. Настоящая работа выполнена на ERα-положительных (ERα – эстрогеновый рецептор α) клетках рака молочной железы MCF-7 и полученных из них сублиниях, устойчивых к ингибитору mTOR рапамицину или антиэстрогену тамоксифену, а также на ERα-отрицательных клетках рака молочной железы MDA-MB-231. Используемые методы включают тест МТТ, транзиторную трансфекцию, иммуноблоттинг, полимеразную цепную реакцию в реальном времени и анализ метилирования с помощью бисульфитного пиросеквенирования.

Результаты. Мы показали, что резистентность клеток рака молочной железы к таргетным и гормональным препаратам связана с подавлением ДНК-метилтрансферазы 3А (DNMT3A) и соответствующими изменениями в метилировании ДНК. Нокдаун DNMT3A приводит к частичной резистентности к обоим препаратам, что демонстрирует ключевую роль подавления DNMT3A в развитии резистентности опухолевых клеток.

Заключение. В целом, полученные результаты свидетельствуют о возможном механизме формирования устойчивости опухолевых клеток к таргетным/гормональным препаратам, основанном на дерегуляции экспрессии DNMT, и демонстрируют прямую связь между подавлением DNMT3A и развитием резистентности.

Ключевые слова: рапамицин, тамоксифен, лекарственная устойчивость, клетки MCF-7, протеинкиназа AKT, ДНК-метилтрансфераза, повторы LINE

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INTRODUCTION

The development of acquired drug resistance of tumor cells is among the key factors limiting the efficiency of antitumor therapy [1–5]. There are various mechanisms which are respondent for the formation of the resistant phenotype of cancer cells, including the activation of ABC (ATP-binding cassette) transporters, mutations of targeted genes, rearrangement of signaling pathways, etc. [6–8]. Among them, the reconstruction of the epigenetic machinery belongs to the main events involved in the progression and maintenance of the low drug sensitivity of tumor cells [9–12]. DNA (de)methylation involved in the different regulation of genes is associated with the progression of tumor cells resistance, including genes encoding ABC transporters, growth signaling proteins, DNA repair enzymes, growth factors and receptors, etc. [13, 14].

DNA methyltransferases (DNMTs) belong to the key enzymes, which are responsible for DNA methylation and epigenetic regulation of gene expression [15, 16]. Several studies demonstrate various changes in the expression or activity of DNMTs in resistant tumors [17–19], however, the mechanism of the precise regulation of DNMTs is still unclear.

Earlier, we have shown that prolonged treatment of MCF-7 breast cancer cells with mammalian target of rapamycin (mTOR) pathway inhibitors, rapamycin or metformin, resulted in the development of resistant clones characterized by constitutive activation of growth-related pathways [20, 21]. Because the activation of bypass growth signaling is among the key features of the acquired hormonal resistance, we proposed the existence of common mechanism respondent for the formation of cell resistance to both mTOR-targeting and hormonal agents.

Here we have shown that the progression of the resistance of breast cancer cells to targeted and hormonal drugs involved the suppression of DNMT3A axis and the respective changes in DNA methylation, and demonstrated the pivotal role of DNMT3A suppression in the progression of the cell resistance. Following studies will delineate the mechanism of DNMT3A suppression in resistant tumors, and highlight the perspectives of the applying of these parameters as an additional criterion for the prognosis of tumor resistance.

MATERIALS AND METHODS

Cell lines and evaluation of antiproliferative activity. The MCF-7 cells (ATCC HTB-22) and MDA-MB-231 cells (ATCC HTB-26™) were cultured at 37 °C and 5 % CO_2 in DMEM medium (PanEco, Russia) containing 4.5 g/l glucose and 10 % fetal bovine serum (HyClone, USA). Prolonged treatment of the parent MCF-7 cells with tamoxifen and rapamycin was used to obtain resistant sublines MCF-7/T and MCF-7/Rap respectively [20, 22]. The MTT assay [23] with modifications as described [24] was used to determine the cell response to the drugs after the treatment of the cells with tamoxifen or rapamycin.

Transfection of small interfering RNA. Scrambled nonspecific siRNA and DNMT3A specific siRNA were purchased from Syntol. Oligonucleotides were dissolved in annealing buffer (50 mM NaCl, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA), annealed at 95 °C and used for transfection with Lipofectamine 2000 (Thermo Fisher Scientific, USA). The following sequences of siRNA were used in the study: scrambled siRNA 5'-UUCUCCGAACGUGUCACGUTT-3', DNMT3A siRNA 5'-GCCAAGGUCAUUGCAGGAATT-3', with corresponding antisense sequences.

Total RNA isolation and quantitative real-time polymerase chain reaction. Total RNA was extracted from cells using TRIzol reagent (Invitrogen, USA) following the manufacturer's instructions. cDNA was synthesized from 1 μg of total RNA by reverse transcription using the iScript™ Advanced cDNA Synthesis Kit (Bio-Rad, USA). Quantitative real-time polymerase chain reaction (RT-PCR) was performed using 5X qPCRmix-HS SYBR (Evrogen, Russia) with the following conditions: initial denaturation for 3 min at 95 °C, followed by 40 cycles at 95 °C for 15 s, at Ta 60 °C for 15 s (Ta – temperature annealing), and at 72 °C for 30 s. All PCR reactions were performed in triplicate,

and gene expression was normalized relative to that of human β-Actin (ACTB), which was used as internal control. Primers used for RT-PCR are listed in table 1. Amplification and data analysis were performed on CFX96 touch RT-PCR Detection System (Bio-Rad, USA); Bio-Rad CFX Manager software v. 3.1 was used for threshold cycle (Ct) value calculations and data analysis. Relative mRNA expression was determined using the ΔΔCt method [25].

Table 1. *Sequence of primers used in real-time polymerase chain reaction*

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
DNMT1	CGACTACATCAA AGGCAGCAACCTG	TGGAGTGGACTTG TGGGTGTTCTC
DNMT3a	TGGCAGGATAG CCAAGTTCAG	GCTGGTCTTT GCCCTGCTTTATG
ACTB	ATGTGGCCGAGGA CTTTGATT	AGTGGGGTGG CTTTTAGGATG

Immunoblotting. Preparation of the cell lysates for immunoblotting was conducted as described previously in [26]. The lysates were separated by 10 % SDS-PAGE, transferred to a nitrocellulose membrane (PerkinElmer, USA), and processed as described earlier [27]. After the treatment with 5 % nonfat milk (Applichem, Germany) the membranes were incubated with primary antibodies (Cell Signaling Technology) overnight at +4 °C. For the standardization of loading, the antibodies against α-tubulin (Cell Signaling Technology, USA) were used; the secondary antibodies corresponding IgGs conjugated with horseradish peroxidase were provided by Jackson ImmunoResearch (USA). The detection was performed using Mruk and Cheng's protocol [28] and an ImageQuant LAS4000 system for chemiluminescence (GE HealthCare, USA). Densitometry for immunoblotting data was performed using ImageJ software (Wayne Rasband). The protocol for densitometry was provided by The University of Queensland with the recommendations from the work [29].

Methylation analysis by bisulfite pyrosequencing. Methylation analysis was carried out using a bisulfite pyrosequencing method. Briefly, genomic DNA was isolated using the ExtractDNA Blood & Cells kit (Evrogen, Russia) and modified with sodium bisulfite using the EZ DNA Methylation-Gold™ Kit (Zymo Research, USA) according to the manufacturer's protocol. Bisulfite treated DNA was amplified with a pair of gene-specific primers using 5x MaSTaqDD PCR master mix (Dialat Ltd., Russia). One of these primers was biotinylated for subsequent immobilisation of a specific DNA-fragment onto a solid support (by biotin/streptavidin coupling). Primers and assays used for pyrosequencing are listed in table 2. The PCR products were sequenced by pyrosequencing PyroMark Q24 (Qiagen, Germany) using a specific sequencing primers (table 2). The obtained sequences were analyzed using PyroMark Q24 Advanced Software, which allows analysis of methylation levels of CpG sites.

Statistical analysis. Each experiment was repeated three times with three technical replicates. Statistical analysis was performed using Microsoft Excel. Results were expressed as mean + SD (standard deviation value) if not stated explicitly. A p-value of <0.05 was considered statistically significant.

RESULTS

DNMT3A expression and cell resistance. The experiments were performed on MCF-7 breast cancer cells and rapamycin-resistant (MCF-7/Rap) and tamoxifen-resistant (MCF-7/T) sublines developed under prolonged treatment of the parent cells with mTOR inhibitor rapamycin or selective estrogen ERα modulator tamoxifen respectively [20, 22]. In a parallel, the study of the tamoxifen-resistant ERα-negative MDA-MB-231 breast cancer cells were performed.

Study of the DNA methylation enzymes showed the significant suppression of DNMT3A protein level in both MCF-7 resistant sublines, whereas the expression of DNMT1 was not changed significantly (fig. 1*a*). Similarly,

Table 2. *Primers and pyrosequencing assays*

Gene	Forward primer (5'-3')	Reverse primer (5'-3')	Sequencing primer (5'-3')	Assay
LINE-1	TGAGTTAGGTG TGGGATATAGT	bio~AAAATCAAAA AATTCCCTTTC	GTTAGGTGTG GGATATAGTTT	YGTGGTGYG TYGTTTTTTAA GTYGGTTT GAAAAGYGTAATA TTYGGGTGGGA
DNMT3A cg03463641	TAGTATTGGGGT TGGGGATAGTAG	bio~ACCTTAACCCTAT AAAACAAAATAACCTC	TAGTATTGGGGT TGGGGATAGTAG	YGTTGGTTTA ATYGYGTYGTA ATTTTTAG
DNMT3A cg21629895	TGGAAGATTTTGT GTGTGTTTATATAT	biotin-AAATCAAAAA CCTAAAACCCTAAAC	TGGAAGATTTTGT GTGTGTTTATATAT	YGTTTTTATTTT TTTATYGTGGGGG TTGTTTTTTTTTTTT ATGGAGYGTTT

Note. *Symbol Y represents C or T according to the IUPAC codes.*

RT-PCR analysis revealed the marked decrease in the level of mRNA DNMT3A in the resistant cells when the level of mRNA DNMT1 was decreased slightly (fig. 1*b*). Similarly to the MCF-7 resistant sublines, the ERα-negative MDA-MB-231 cells were characterized with the marked decrease in the protein and mRNA level of DNMT3A when the DNMT1 level was even slightly increased (fig. 1*a*, *b*).

Cell resistance and DNA methylation. To investigate the effect of DNMT3A suppression on the global change in DNA methylation, we examined the methylation level of long interspersed nucleotide element 1 (LINE-1). LINE-1 is a major genetic element, making up ~17 % of the entire genome [30]. CpG sites located within LINE-1 and their methylation levels correlate with the global methylation status of genomic DNA and therefore often used as a surrogate marker for assessing global DNA methylation alterations [31].

The analysis of LINE-1 methylation in the resistant sublines revealed a slight decrease in that in tamoxifen- and rapamycin-resistant cells as well as in the MDA-MB-231 cell line (not significant) (fig. 2) supporting the repression of DNMT3A activity in the resistant cells.

In addition, we analyzed the methylation of regulatory sequences, a CpG-island (CGI) in the promoter of the DNMT3A gene and in transitional areas, termed shores, to elucidate potential mechanisms of suppression of DNMT3A expression in resistant cells. There were no significant changes in the DNA sequence methylation levels in the CGI and S-shore regions in the MCF-7/T and MCF-7/Rap resistant cells compared to the parental cell line. According to The Cancer Genome Atlas (TCGA) (breast cancer data collection, BRCA), increased methylation of S-shore region (genomic coordinates chr2:25565794–25565842, hg19/Human) is associated with reduced DNMT3A expression. Thus, DNA methylation, at least of the S-shore region of DNMT3A gene, does not seem to be responsible for suppression of DNMT3A expression in resistant cells. In contrast, we found increased methylation in the CpG-island in DNMT3A promoter in MDA-MB-231 cells, which may be one of the reasons for the decreased DNMT3A activity in these cells (fig. 2).

Influence of DNMT3a knockdown on the cell resistance. As can be seen in fig. 3, knockdown of DNMT3A by siRNA resulted in the progression of partial resistance of MCF-7 cells to both tamoxifen and rapamycin underlining the direct association between DNMT3A suppression and formation of the resistant phenotype.

Totally, we have revealed the pivotal role of suppression of DNMT3A in the formation of the cell resistant phenotype. Namely, we have described the suppression of DNMT3A axis in the rapamycin- and tamoxifen-resistant cells, and demonstrated the similar progression of partial cell resistance under DNMT3A knockdown; we have shown that suppression of DNMT3A axis was associated with the decrease in the DNA methylation. The following studies will delineate the prevalence of DNMT3A suppression among the resistant tumors, and reveal the possible applying of these parameters as the criteria of the cell resistance to targeting drugs.

DISCUSSION

The high level of tumor cell resistance to drug treatment is among the key factors determining the relatively low efficiency of anticancer therapy [2]. As known, long-term drug treatment is accompanied with the progression of acquired drug resistance of tumors, and, in the case of the specific targeting drugs, may be based on the rearrangement

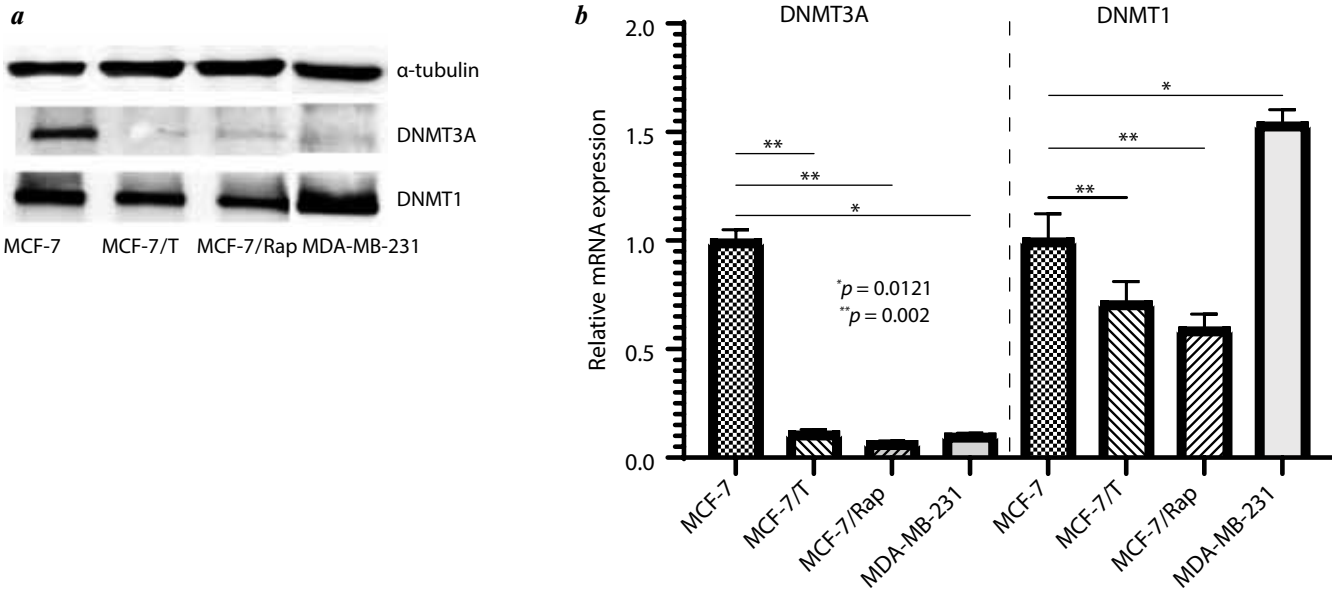

Fig. 1. *DNMTs protein and RNA expression levels in the cell lines: a – Western blotting of the protein samples of MCF-7, MCF-7/T, MCF-7/Rap and MDA-MB-231 cells. Densitometry was used to quantify changes in protein expression; b – RT-PCR analysis of the expression of DNMT3A and DNMT1 in MCF-7, MCF-7/T, MCF-7/Rap and MDA-MB-231 cells*

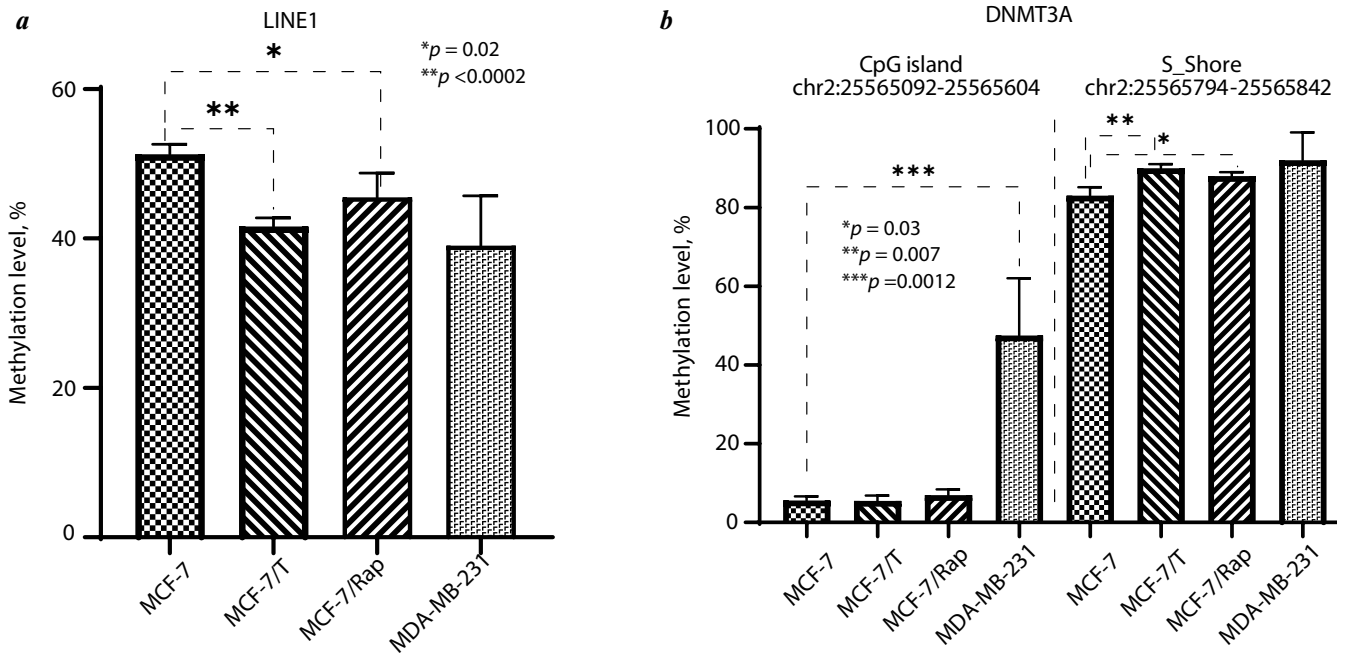

Fig. 2. *Methylation levels in the studied cell lines: a – average methylation level of the six CpG dinucleotides in the LINE-1 region in MCF-7, MCF-7/Rap, MCF-7/T and MDA-MB-231 cells; b – average methylation of the four CpG dinucleotides within CpG-island (CGI) in the promoter of the DNMT3A gene and of the three CpGs in the adjacent N-shore sequence*

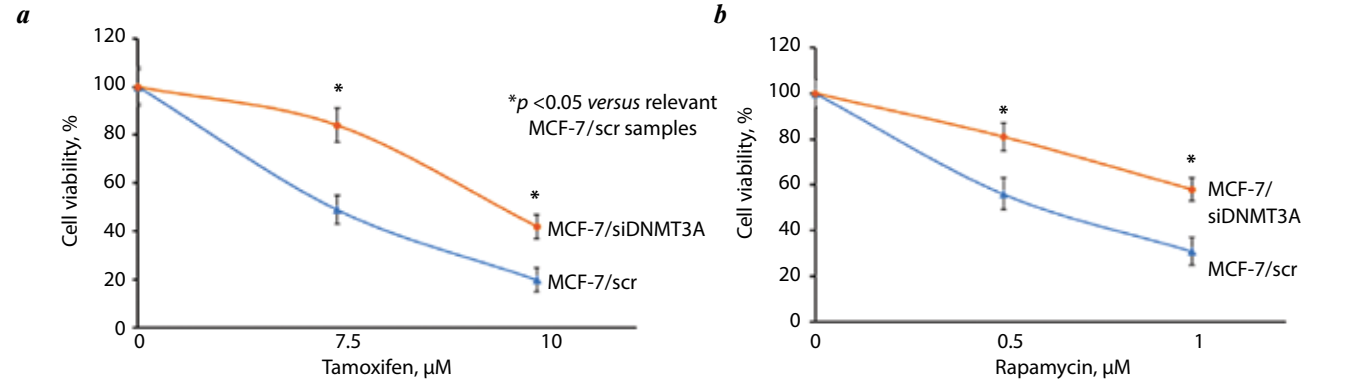

Fig. 3. *The viability of MCF-7/scr and MCF-7/siDNMT3A cells after tamoxifen (a) and rapamycin (b) treatment. Data represent mean value ± standard deviation of three independent experiments*

signaling pathways respondent for the regulation of cell proliferation [3].

Similar to targeted drug resistance, progression of cell resistance to hormonal drugs possesses the common features, i.e. the rearrangement of signaling pathways and activation of bypass estrogen-independent pathways resulting in the restoration of cell proliferation [6]. A number of works, including our studies, described the changes in the profile of proteins and nucleic acids in the resistant cells [5, 20, 32–34], revealed microRNAs involved in the development of the resistance of tumor cells [35], and demonstrated the participation of exosomes in the transfer of hormonal resistance [36].

It should be noted that the mechanism of maintaining of cell signaling rearrangement and, in particular, the significance of epigenomic changes, i.e. DNA methylation, in the development of tumor cells resistance are currently being actively studied using various experimental models [37]. ERα was found to regulate gene expression in breast cancer cells in part via DNA methylation whereas the loss of ERα results in the progression of estrogen resistance associated with the hypomethylation and overexpression of mitogen signaling genes [13]. Totally, the comparative analysis of the DNA methylation profile of the estrogen-sensitive and resistant breast cancer cells revealed number of genes hyper- or hypomethylated in the resistant cells [13, 14]. Among the recently studied genes some of histone variants overexpressed in the resistant cells [38], hypomethylation of Lactate dehydrogenase genes in the MCF-7 resistant clones [39] and some others. Earlier, using the panel of various MCF-7 resistant derivates we have identified six coding genes, PRKCZ, TRAPPC9, ASIC2, C2CD4a, ZNF787 and CRTAC1, differentially methylated in the resistant cells [40].

In the present work, the expression and activity of the key DNA methyltransferases in the MCF-7 resistant sublines was analyzed. As revealed, both tamoxifen-resistant and rapamycin-resistant cells were characterized by common features – constitutive suppression of DNMT3A associated with hypomethylation of numerous DNA regulatory sequences. Knockdown of DNMT3A in parental MCF-7 cells was accompanied with the development of partial cell resistance to rapamycin and tamoxifen, indicating the involvement of DNMT3A in the formation of cell response to the tested drugs. The association of DNMT3A suppression with cell resistance was confirmed in the experiments on the ERα-negative MDA-MB-231 breast cancer cells showing the low level of DNMT3A expression in MDA-MB-231 cells compared with the estrogen-dependent MCF-7 cells.

CONCLUSION

Totally, the results obtained highlight the possible mechanism of tumor cell resistance to targeting/hormonal drugs based on the deregulation of DNMTs expression and demonstrate direct connection between DNMT3A suppression and resistance progression; further studies are required to identify the factors involved in DNMT3 deregulation in the resistant cells.

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