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Proceeding Paper

The Phenomenon of the Cross-Resistance of Breast Cancer to Target and Hormonal Drugs: The Role of Epigenetic Reconstruction †

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Abstract: The rearrangement of molecular pathways and the activation of bypass signaling determine the progression of tumor cell resistance to various drugs that specifically block target signaling proteins. The present work was performed on the MCF-7 breast cancer cells and established sublines, resistant to mTOR inhibitor rapamycin or antiestrogen tamoxifen, developed under prolonged cell treatment with rapamycin or tamoxifen, respectively. We have shown that both resistant sublines demonstrate the cross-resistance to rapamycin and tamoxifen and are characterized with the common signaling changes, namely—blocking of the estrogen receptor α (ERα) transcriptional activity and constitutive activation of Akt signaling. Analysis of the epigenetic machinery revealed the drastic suppression of the level of DNA methyltransferase 3A (DNMT3A) in both the resistant sublines that were correlated with the demethylation of the LINE-1 repeats. Knockdown of the DNMT3A via siRNA results in the progression of partial resistance of MCF-7 cells to both tamoxifen and rapamycin, supporting the important role of DNA methylation in the formation of the resistant phenotype. Totally, the results obtained highlight the possible mechanism of the tumor cell resistance to targeting/hormonal drugs based on the rearrangement of DNA methylation profile and activation of the bypass signaling pathways.

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

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1. Introduction

The development of acquired drug resistance of tumor cells is among the key factors limiting the efficiency of antitumor therapy. There are various mechanisms responsible for the formation of the resistant phenotype of cancer cells including the activation of ABC (ATP-binding cassette) transporters, mutations of the targeted genes, rearrangement of the signaling pathways, etc. [1–3]. Among them, the reconstruction of epigenetic machinery belongs to the main events involved in the progression and maintenance of the low drug sensitivity of tumor cells [4,5].

Earlier, we have shown that prolonged treatment of MCF-7 breast cancer cells with mTOR pathway inhibitors, rapamycin or metformin, results in the development of the resistant clones characterized with constitutive activation of growth-related pathways [6]. Because the activation of bypass growth signaling is among the key features of the acquired hormonal resistance, we proposed the existence of the common mechanisms that are responsible for the formation of cell resistance to both mTOR-targeting and hormonal agents.

2. Methods

2.1. Cell Cultures and Evaluation of Antiproliferative Activity

The MCF-7 cells (ATCC HTB-22) were cultured at 37 °C and 5% CO_2 in DMEM medium (PanEco: Moscow, Russia) containing 4.5 g/L glucose and 10% fetal bovine serum (HyClone, Cytiva: Marlborough, MA, USA)). Prolonged treatment of the parent cells with tamoxifen and rapamycin, respectively, was used to obtain resistant sublines MCF-7/T and MCF-7/Rap [6]. The MTT assay [7] was used to determine the cell response to the drugs after the treatment of the cells with tamoxifen or rapamycin.

2.2. Transient Transfection and Measurement of Reporter Gene Activity

To measure estrogen receptor (ERα) transcriptional activity, the transfection of plasmids containing estrogen-responsive elements that controlled the luciferase gene was performed as described previously [8]. The cells were co-transfected with β-galactosidase plasmids; the relative luciferase activity was calculated as the luciferase to galactosidase ratio.

2.3. 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: Waltham, MA, USA). The following sequences of siRNA were used in the study: scrambled siRNA 5′-UUCUCCGAACGUGUCACGUTT-3′ and DNMT3A siRNA 5′-GCCAAGGUCAUUGCAGGAATT-3′ with corresponding antisense sequences.

2.4. Immunoblotting

Preparation of the cell lysates was conducted as described previously [6]; they were separated using 10% SDS-PAGE, transferred to a nitrocellulose membrane (PerkinElmer: Waltham, MA, USA), and processed as described earlier [9]. After the treatment with 5% nonfat milk (AppliChem: Darmstadt, Germany), the membranes were incubated with primary antibodies (Cell Signaling Technology: Danvers, MA, USA) overnight at +4 °C. For the standardization of loading, the antibodies against α-tubulin (Cell Signaling Technology) were used; the secondary antibodies corresponding to IgGs conjugated with horseradish peroxidase were provided by Jackson ImmunoResearch (Ely, UK). The detection was performed using Mruk and Cheng's protocol [10] and an ImageQuant LAS4000 system for chemiluminescence (GE HealthCare: Chicago, IL, USA).

2.5. Bisulfite Pyrosequencing for LINE-1 Methylation Analysis

LINE-1 methylation analysis was carried out using a bisulfite pyrosequencing method. Briefly, genomic DNA was isolated using the ExtractDNA Blood & Cells kit (Evrogen: Moscow, Russia) and modified with sodium bisulfite using the EZ DNA Methylation-Gold™ Kit (Zymo Research: Tustin, CA, USA) according to the manufacturer protocol. Bisulfite-treated DNA was amplified with the forward (5′-TGAGTTAGGTGTGGGATATAGT-3′) and biotinylated reverse primers (5′-bio-AAAATCAAAAAATTCCCTTTC-3′) using 5X MaSTaqDD PCR master mix (Dialat Ltd.: Moscow, Russia). The PCR products were sequenced via pyrosequencing with PyroMark Q24 (Qiagen: Hilden, Germany) using a specific sequencing primer 5′-GTTAGGTGTGGGATATAGTTT-3′ with the analyzed sequence: 5′-YGTGGTGYGTYGTTTTTTAAGTYGGTTTGAAAAGYGTAATATTYGGGTGGGA-3′. The obtained sequences were analyzed using PyroMark Q24 Advanced Software (Qiagen: Hilden, Germany), which allows the analysis of the methylation levels of CpG sites.

2.6. Statistical Analysis

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

3. Results and Discussion

The experiments were performed on the 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 rapamycin or tamoxifen, respectively. The study of the cell sensitivity to the indicated drugs revealed the high level of cross-resistance to rapamycin and tamoxifen in both the sublines (Figure 1).

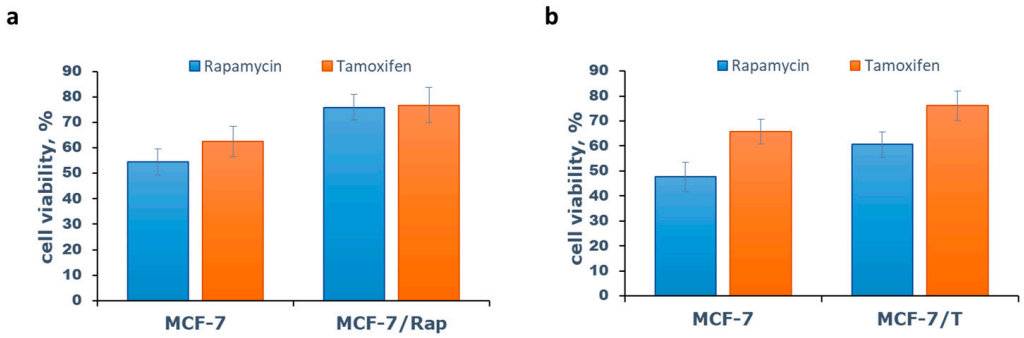

Figure 1. Cross-resistance of MCF-7/Rap (**a**) and MCF-7/T (**b**) cells to rapamycin and tamoxifen. The cells were treated with 2 μM rapamycin or 3 μM tamoxifen for 3 days with subsequent analysis of the number of viable cells using the MTT test. Data represent mean value ± S.D. of three independent experiments. A total of 100% was set as the viability of cells treated with the vehicle control.

The resistant sublines were characterized with the weakened ERα activity along with constitutive activation of Akt signaling, when the expression of mTOR signaling proteins was not affected (Figure 2a,b).

The analysis of the epigenetic machinery revealed the drastic suppression of the level of DNA methyltransferase 3A (DNMT3A) in both the resistant sublines whereas the expression of DNA methyltransferase 1 was not changed (Figure 2a). To investigate the effect of suppressing DNMT3A expression in resistant sublines on the global change in DNA methylation in the studied cells, 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 [11]. CpG sites located within LINE-1 and their methylation levels correlate with the global methylation status of genomic DNA and are therefore often used as a surrogate marker for assessing global DNA methylation alterations [12]. LINE-1 methylation was analyzed in parental and resistant MCF-7 sublines via bisulfite pyrosequencing. In three independent experiments, we showed that the LINE-1 methylation level was significantly lower in tamoxifen- and rapamycin-resistant cells when compared to parental MCF-7 cells (Figure 2c).

Totally, the results obtained highlight the possible mechanism of the tumor cell resistance to targeted/hormonal drugs based on the rearrangement of the DNA methylation profile and changes in the epigenetic regulation of cell signaling. Importantly, the changes in DNA methylation profile in the hormone-resistant or targeted drug-resistant cells were demonstrated with various cell models [4,13,14]; however, the precise mechanisms responsible for such alterations are still under investigation. We suggest that further studies are required for the identification of key signaling pathways mediating the effect of DNMT3A suppression and involved in the progression of cancer drug resistance.

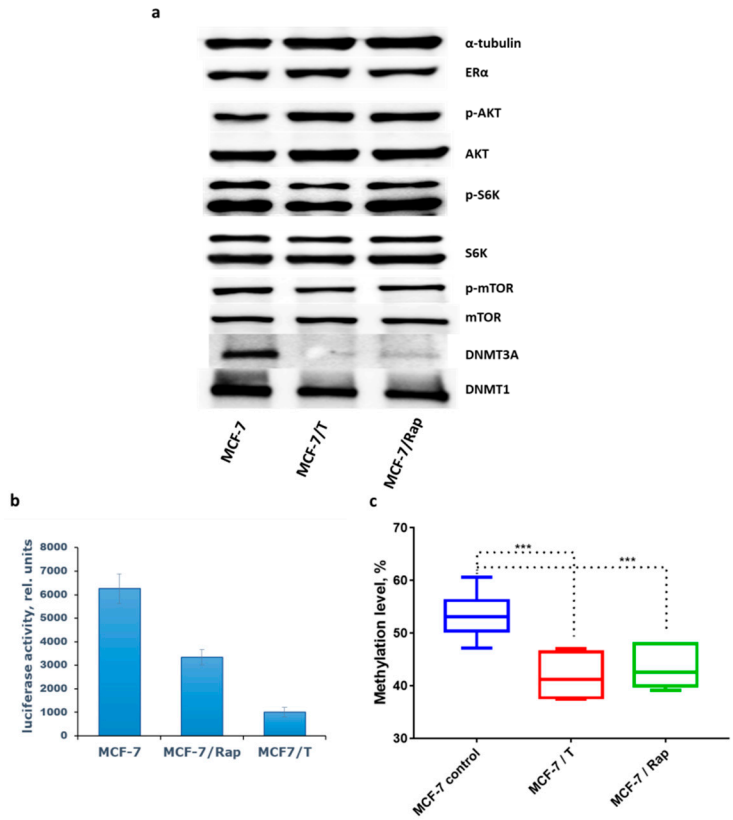

Figure 2. (**a**) Western blotting of the protein samples of the MCF-7, MCF-7/Rap, and MCF-7/T cells. The blot represents the results of one of the three similar experiments. Expression of α-tubulin, ERα, Akt/p-Akt, S6K/p-S6K, mTOR/p-mTOR, DNMT3A, DNMT1 was studied. (**b**) Reporter analysis of the ERα transcriptional activity. The relative luciferase activity was calculated in arbitrary units as the ratio of the luciferase to the galactosidase activity. Data represent the mean value ± S.D. of three independent experiments. (**c**) The average methylation level of the six CpG dinucleotides in the LINE-1 region. *** p-value < 0.0006. To further investigate the role of DNMT3A in the formation of cell-resistant phenotypes, the knockdown of the DNMT3A via siRNA was performed. As revealed, transfection of siRNA DNMT3A results in the progression of partial resistance of MCF-7 cells to both tamoxifen and rapamycin (Figure 3).

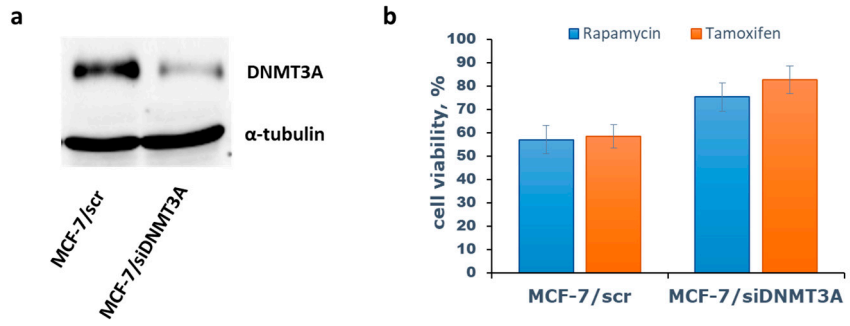

Figure 3. (**a**) Western blotting analysis of DNMT3A in the MCF-7 cells after siRNA DNMT3A transfection. Scrambled siRNA was used as a control. The blot represents the results of one of the three similar experiments. (**b**) The sensitivity of MCF-7/scrambled and MCF-7/siDNMT3A cells to rapamycin (2 μM) and tamoxifen (3 μM). Data represent the mean value ± S.D. of three independent experiments.

4. Conclusions

Taken together, the results presented demonstrate the existence of the common mechanisms responsible for the activation of the bypass signaling pathways in the cells resistant to

mTOR targeting or hormonal drugs, and revealed the involvement of the DNA methylation enzymes in the formation of the cell resistant phenotype.

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