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EFFECT OF 5-AZACYTIDINE ON miRNA EXPRESSION IN HUMAN BREAST CANCER CELLS WITH DIFFERENT SENSITIVITY TO CYTOSTATICS

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Aim: To analyze expression of miRNA in human breast cancer cells, sensitive and resistant to cisplatin and doxorubicin, and to explore possible modification of drug sensitivity via treatment of cells with 5-azacytidine (5-aza), a demethylating agent. *Materials and Methods:* The study was performed on wild-type MCF-7 cell line (MCF-7/S) and its two sublines MCF-7/Dox and MCF-7/DDP resistant to doxorubicin and cisplatin, respectively. Cells were treated with 5-aza, cisplatin, doxorubicin and their combinations. Relative expression levels of miRNA-221, -200b, -320a, -10b, -34a, -122 and -29b were examined, using qRT-PCR. The MTT assay was used to monitor cell viability. *Results:* We compared miRNA expression profiles in MCF-7/S and drug resistant MCF-7/Dox and MCF-7/DDP cells. Changes of miRNA-221, -200b, -320a, -10b, -34a, -122 and -29b were observed in both resistant cell lines. The most significant differences were found for miRNA-200b (decreased in 50.0 ± 2.6 and 63.0 ± 3.1 times for MCF-7/Dox and MCF-7/DDP cells, respectively) and for oncogenic miRNA-221 levels (increase in 62.0 ± 5.7 times for MCF-7/Dox and 83.8 ± 7.2 times for MCF-7/DDP cells). 5-aza treatment caused an increase of miRNA-10b, -122, -200b levels in MCF-7/S cells, miRNA-34a, -10b, -122, -200b and -320a levels in MCF-7/Dox cells and miRNA-34a, -10b, -200b and -320a levels in MCF-7/DDP cells. Pretreatment of all studied lines with 5-aza resulted in the increase of their sensitivity to studied cytostatics. In particular, the IC50 of doxorubicin decreased by 2-, 4- and 3-fold for cell lines MCF-7/S, MCF-7/Dox and MCF-7/DDP cells, respectively, and IC50 of cisplatin in studied cultures decreased by 3-, 2- and 1.5-fold, respectively. *Conclusions:* It was shown that use of 5-aza can modify sensitivity of breast cancer cells to cytotoxic drugs not only by it's demetylation effect, but also by changes in expression of miRNAs, involved in cell proliferation, migration and drug resistance development.
Key Words: MCF-7, drug sensitivity, epigenetics, miRNA, methylation.

Oncological diseases remain one of the most common causes of death. Genetics studies accelerated cancer research significantly. Moreover, identification of oncogenes and tumor suppressor genes was a breakthrough for the development of specific drugs to treat cancer, but has not lead to creation of a panacea. Recent studies showed that in addition to genetic disorders, the development of cancer is triggered by epigenetic changes. Changes in DNA methylation and miRNA expression are important factors, controlling oncogenesis and drug resistance development [1, 2].

DNA methylation in oncogenesis. DNA methylation is a key epigenetic modification that often leads to silencing on the gene transcription. Cancer cells are characterized by abnormal DNA methylation patterns, accompanied by global hypomethylation and gene-specific hypermethylation, as a rule [2].

DNA methylation occurs when DNA methyltransferases (DNMT1, DNMT3a and DNMT3b) add the methyl group to the cytosine residue of DNA, leading to the 5-methylcytosine synthesis. DNA methylation occurs mainly in areas rich of so called CpG-islands. CpG-islands are found in promoter regions of majority of genes, and negative correlation between promoter methylation and gene expression is already well known. Hypermethylation of CpG-islands in promoter region almost always causes the gene silencing, regardless on gene mutations. During breast cancer (BC) development, changes of DNA methylation are responsible for inactivation of large number of genes, including genes that control the cell cycle, steroid receptor genes, genes associated with metastasis, resistance to drugs, etc [3].

Changes in DNA methylation lead to genetic instability: 5-methylcytosine serve as a transition mutations site — through hydrolytic deamination of 5-methylcytosine becomes thymine. This mutation is frequently observed in such genes as *TP53*, *RB1* and *HRAS*. Also, epigenetic inactivation of critical genes through promoter hypermethylation is a prerequisite for genetic instability — for example, methylation of *MLH1*, which participates in mismatch reparation.

Furthermore, methylation of promoter CpG-islands and inactivation of glutathione-S-transferase leads to the accumulation of free radicals and further DNA damage. Besides some regional gene hypermethylation, genome hypomethylation is one of the important characteristics of the tumor, which also contributes to genetic instability [4].

It has been suggested that aberrant DNA methylation can affect the sensitivity of tumor cells to anticancer agents altering the expression of genes that are involved in the response to the impact of xenobiotics. DNA hypermethylation may play an important role in development of drug resistance phenotype by inactivation of genes that are involved in the cytotoxicity reactions [5].

Significant reduction in the accumulation of methotrexate and other drugs in human hepatoma and

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Abbreviations used: 5-aza – 5-azacytidine; BC – breast cancer; DDP – cisplatin; DNMT – DNA methyltransferases; Dox – doxorubicin; EMT – epithelial-mesenchymal transition; MDR – multidrug resistance.

epidermal carcinoma cells resistant to cisplatin (DDP) was demonstrated as the result of silencing of folate-binding gene *FBP*. Restoration of this gene expression after treatment with a demethylating agent 2-deoxy-5-azacytidine (5-aza) proved the hypermethylation of the *FBP* promoter in drug resistant cells.

Similar processes occur in testicular tumors; *in vivo* DDP induces promoter hypermethylation *de novo*. *In vitro* acquired resistance to DDP causes changes in the expression of a number of genes, but even in high-resistant cells the expression of tumor suppressor genes is restored after treatment with demethylating agents [6].

It was found that expression of several hundred genes decreased in DDP resistant cell lines and it can be restored by DNMT inhibitors [6–8].

Concluding, the aberrant DNA methylation is a common event in development of drug resistance in cancer cells. Moreover, CpG-methylation leading to gene silencing may play an important role in resistance to cytostatic chemotherapy.

miRNAs in cancer development. miRNAs are small (18–25 nucleotides) noncoding RNAs, negatively regulating expression of target genes through initiation of cleavage of their mRNA. miRNA binds to the target complementary mRNA, resulting in cleavage and/or de-adenylation of the latter, or obstructs ribosome movement [2].

Usually miRNA genes are transcribed by RNA polymerase II. Formed large transcript consists of several hundred base pairs, named pri-miRNA, containing 5'Cap and poly-A-tail [9].

Then double stranded structures in pri-miRNA are recognized by protein DGCR8 (also called "Pasha"), which forms microprocessor protein complex with an enzyme Drosha that possesses RNAse III domain. In the result, approximately 70 bp-length pre-miRNA is formed with the shape of a stem-loop-structure (hairpin). This pre-miRNA is recognized by Exportin-5 and with the participation of Ran-GTPase is transported to the cytoplasm [10, 11].

In the cytoplasm, pre-miRNA is digested by enzyme Dicer (RNAse III) into a duplex of 22 nucleotides length. Dicer initiates the formation of RNA-induced silencing complex (RISC). RISC protein family consists of the Argonautes (Ago), with endonuclease activity towards mRNA that is complementary to microRNA, incorporated in RISC [12].

RISC is responsible for gene silencing, due to binding to 3'-UTR of the mRNA and repressing its translation [13].

Thus, miRNAs can inhibit the expression of target genes through a variety of mechanisms by preventing protein translation without changing of the mRNA level or by mRNA degradation. miRNA genes are often located in so-called "fragile sites" of chromosomes, where often occur deletions, insertions, DNA strand breaks (single and double), translocations, transitions, amplification etc., making them weak link in the formation of mutated phenotype [2, 15].

Epigenetic mechanisms in development of drug resistance. As was mentioned above, altered expression of genes, leading to drug resistance development can be regulated not only by genetic alterations, but also by epigenetic mechanisms. Thus, correction of methylation disturbances and microRNAs expression can be used for correction of cancer cells sensitivity [1, 15].

While miRNAs are involved in regulation of many cellular processes, from zygote polarization to cell death, upon development of drug resistance only miRNAs, responsible for cell proliferation, migration, etc. showed altered expression.

Many miRNAs are described as oncogenic — they negatively regulate expression of tumor suppressor genes, for example, *RB1*, a cell cycle regulator and *PTEN* (phosphatase and tensin homolog) an inhibitor of cell survival/growth. *PTEN* is the target of several different miRNA — miRNA-21, -216, -221, and -144. Such variety of regulatory pathways can be used to overcome the drug resistance by maintenance of expression of tumor suppressor [1, 16].

Also, there are tumor suppressor miRNAs. Mutations in their genes and/or epigenetic silencing of expression can cause cancer progression, and also increasing in invasive and migration properties. For example, miRNA-200b negatively regulates family of ZEB proteins, responsible for epithelial-mesenchymal transition (EMT). Decrease in miRNA-200b expression is one of EMT triggers in BC cells [1].

It is well documented that resistance of MCF-7 cells to doxorubicin (Dox) is mediated by increase of *MDR1* (multidrug resistance) gene copy numbers. Transfection of miRNA-451 causes increase in sensitivity to this cytostatic, as result of inhibition of MDR1 protein translation by degradation of mRNA [17].

As was mentioned already, development of drug resistance is often accompanied by disturbances in DNA methylation. For example, total DNA methylation is lower in MCF-7 cells, compared with normal breast cells. It was proposed that resistance of cancer cells to Dox and DDP was developed due to alterations in methylation pattern of DNA in the specific regions, leading to differentially methylated genes. Such changes in methylation of promoter CpG-islands, responsible for the specificity of transcription factors are essential not only for expression of genes, but also for genome stability [18].

Therefore, the aim of the present work was to analyze expression of miRNA in human BC cells sensitive and resistant to DDP and Dox, and explore possible modification of drug sensitivity via treatment of the cells with 5-aza, a demethylating agent.

MATERIALS AND METHODS

Three sub-lines of breast carcinoma MCF-7 cells (obtained from invasive breast ductal carcinoma), namely MCF-7/S, sensitive to cytostatics; MCF-7/Dox — resistant to Dox; and MCF-7/DDP — resistant to DDP were used in our study. Cells were provided by the Bank of human and animal tissues lines

at R.E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology of NAS of Ukraine and described earlier in [19].

All cells were grown in Dulbecco Modified Eagles Medium (DMEM, Sigma), supplemented with recombinant human insulin (0.01 mg/ml) and 10% fetal bovine serum. All cultures were grown in humidified atmosphere with 5% CO_2 at 37 °C.

Measurement of cell viability, using the MTT assay. Ability of living cells to reduce tetrazole (MTT, solution of the yellow color) into purple crystalline formazan by the mitochondrial enzymes allow us to estimate cell viability. The resulting intracellular formazan can be solubilized and quantified, using spectrophotometry [20].

For this purpose, cells were cultivated in 96-well plates in DMEM, supplemented with insulin (see above) for 24 h. The 5-aza, Dox, and DDP were added to media at different concentrations.

After 24 h, 10 µl of the MTT dye solution (Sigma, USA) (5 mg/ml in phosphate buffer saline) was added to the cells; the cells were incubated at the same conditions for 3 h. After centrifugation (1500 rpm, 5 min) the supernatant was removed. 100 µl of dimethyl sulfoxide (Serva, Germany) was added to each well, to dissolve formazan. The absorption was measured, using a multiwell spectrophotometer (STAT FAX 2100, USA) at a wavelength of 540 nm.

Cytotoxicity of studied agents was studied using IC50 and IC30 values [21].

Total RNA isolation. Total RNA extraction was performed, using Ribozol RNA Isolation Kit (Amplisens, Russia). Concentration of RNA was measured, using NanoDrop 2000c Spectrophotometer (Thermo Scientific, USA). The purity of isolated RNA was controlled, analyzing the ratio of OD at 260/280 nm. RNA was dissolved in TE buffer and stored at −20 °C.

Single-stranded cDNA was synthesized from 100 ng of total RNA, using TaqMan® MicroRNA Kit for reverse transcription.

Real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR). Preparation of reverse transcription reaction mix was performed according to manufacturer's protocol. Reverse transcription was performed at a "Tertsik" ("DNA Tehnologiya", Russian Federation) thermal cycler. qRT-PCR was performed on Applied Biosystems 7900HT Fast Real-Time PCR System using TaqMan® MicroRNA primers and manufacturer's protocol.

Small nucleolar RNA RNU48 was used as an endogenous control for normalization of miRNA expression. Relative expression of the studied miRNAs was identified by comparative Ct method [22]. Experiments were performed in triplicates for each line, and PCR was performed three times for each sample. Expression differences between the studied miRNA levels relative to control was calculated by the formula:

$$Fold\ change = 2^{-\Delta\Delta Ct}\ [22],$$

where ΔCt (target — control) is equal to the difference between threshold cycles for miRNA (target) and the threshold cycle for RNU48 (control) (ΔCt (target — control) = Ct target − Ct control). ΔΔCt = ΔCt (experiment) − ΔCt (control).

Experimental data were analysed using the Student's t-test. P-values less than 0.05 were considered statistically significant. Statistical analysis of the obtained data was performed, using the STATISTICA 6.0 software.

RESULTS AND DISCUSSION

The analysis of miRNA expression showed that the development of resistance to cytotoxic drugs is accompanied by a significant decrease in the expression of tumor suppressive miRNA-200b: in cells resistant to Dox — 50 fold decrease; in cells resistant to DDP — 63 fold decrease. For the miRNA-320a downregulation was 8.5 fold and 3.9 folds, respectively. In addition, the increased expression of oncogenic miRNA-221 was observed — 62 fold and 83.8 folds, respectively. miRNA-10b and miRNA-34a expression changed differently in MCF-7/Dox (reduced) and MCF-7/DDP (increased) cell lines (Fig. 1).

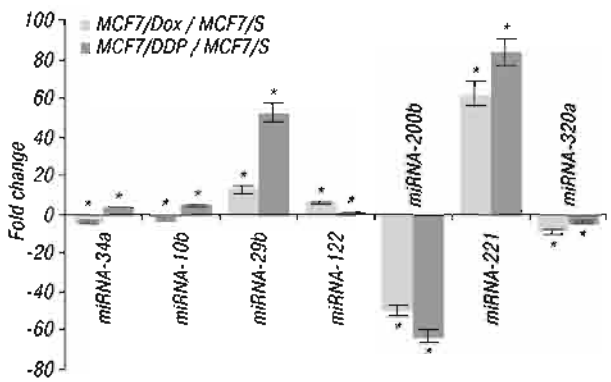

Fig. 1. Fold change of miRNAs expression in resistant to Dox and DDP MCF-7 cells comparatively to sensitive cell line; *p < 0.05. Average ± SD values are shown

5-aza irreversibly binds to DNMT1 during DNA replication and prevents methylation, reduces the number of active DNMT1 molecules in cell and provides a cytotoxic DNA adducts formation [23, 24].

The depletion of *DNMT1* causes progressive loss of methylation upon DNA replication. 5-aza-induced hypomethylation affects the entire DNA, and also CpG-islands in the gene promoter regions; hypomethylation of promoters is often associated with induction of transcription [25, 26]. For example, due to 5-aza treatment, loss of promoter methylation and induction of transcription was shown for *BRCA1*, the tumor suppressor gene that is critical in the ethiology of hereditary and sporadic BC [18, 27].

The web resources (at *http://refgene.com and https://genome.ucsc.edu*) to analyze DNA segments at a distance of 1.5 kb in up- and downstream regions from the transcription start site was used to find CpG-islands in promoter regions of miRNA-221, -200b, -320a, -34a, -10b, -122 and-29b. Several CpG-islands were found in areas, where their methylation can prevent binding of transcription factors to the promoter.

Summarizing, the promoters of miRNA-200b, -10b and -320a are characterized by a high CpG density, that

makes these genes more sensitive to demethylation influence by described mechanism.

As shown in Table 1, miRNA-10b, -122, -221, and -320a showed increased expression in MCF-7/S cells upon 5-aza treatment. The most considerable changes were detected in miRNA-200b expression (2.55 fold increase), and this might lead to inhibition of EMT, metastasizing, proliferation and increase susceptibility to xenobiotics due to its annotated targets [1].

Table 1. Fold change of miRNAs expression after treatment of MCF-7 cells with 5-aza, sensitive and resistant to cytostatics**

miRNA	MCF-7/S + 5-aza	MCF-7/Dox	MCF-7/Dox + 5-aza	MCF-7/ DDP	MCF-7/DDP + 5-aza
miRNA-34a	1.2 ± 0.1	−4.0±0.2*	1.3 ± 0.2	4.0 ± 0.1*	9.3 ± 0.7*
miRNA-10b	2.1 ± 0.1*	−2.0±0.2*	1.3 ± 0.1	5.0 ± 0.9*	8.3 ± 1.3*
miRNA-29b	−1.1 ± 0.2	13.0±0.2*	10.2 ± 0.9*	52.4 ± 5.3*	50.2 ± 7.4*
miRNA-122	2.5 ± 0.1*	6.0 ± 0.4*	7.0 ± 1.0*	1.5 ± 0.3*	1.9 ± 0.4*
miRNA-200b	2.55 ± 0.2*	−50.0±2.9*	−45.1±5.4*	−63.0±3.9*	−59.1 ± 5.3*
miRNA-221	1.7 ± 0.1*	62.0±4.1*	63.1 ± 7.1*	83.8±8.9*	70.1 ± 9.6*
miRNA-320a	1.89 ± 0.1*	−8.5±1.3*	−6.1±0.9*	−3.9 ± 1.5*	1.7 ± 0.3*

Note: *p < 0.05 compared with MCF-7/S cell culture; ** normalized to the values obtained for the MCF-7/S cells without treatment. Average ± SD values are shown.

In Dox-resistant cells upon 5-aza treatment, increase in expression levels of miRNA-34a, -200b and -320a was observed — 2.4, 2.9 and 3.3 folds, respectively, while the expression of miRNA-10b and -122 increased only by half compared with control culture.

The 5-aza treatment caused enhanced expression of miRNA-34a and -10b up to levels typical for the sensitive line. In addition, there was a tendency of differences in reduction fold between sensitive and resistant cultures to Dox; miRNA-122 showed diminished expression, while and miRNA-200b and -320a expression was elevated. However, changes showed different levels, in comparison with sensitive cells.

In MCF-7/Dox cells upon 5-aza treatment, expression of miRNAs, responsible for apoptosis, sensitivity to cytotoxic drugs, cell proliferation and metastasizing, was elevated.

DDP resistant cells were characterized by the following changes: the level of miRNA-320a increased 2 fold, miRNA-10b — 3.32 fold, miRNA-200b — 4 fold, miRNA-34a — 5 fold, in comparison with the control cells.

In MCF-7/DDP cells, in comparison with the sensitive culture the increase of miRNA-320a, up to the level in MCF-7/S cells, enhanced miRNA-200b expression, and reduced oncogenic miRNA-221 level were observed. Noteworthy, miRNA-10b, which is responsible for invasion and metastasis, was expressed at the higher level (Fig. 2).

Changes in drug sensitivity in MCF-7/S, MCF-7/Dox, and MCF-7/DDP cells upon the treatment with 5-aza. The 5-aza is often used in combination with DDP, cyclophosphamide, and other drugs used in the anti-cancer treatment, in particular, in case of neuroblastoma, rhabdomyosarcoma in children, and also certain solid tumors [29]. Chekhun et al. [26] showed the crucial role of epigenetic regulation in acquisition of drug resistance by MCF-7 cells. DNA-methylation disturbances are one of the most frequent causes of DDP and Dox resistance in these cells.

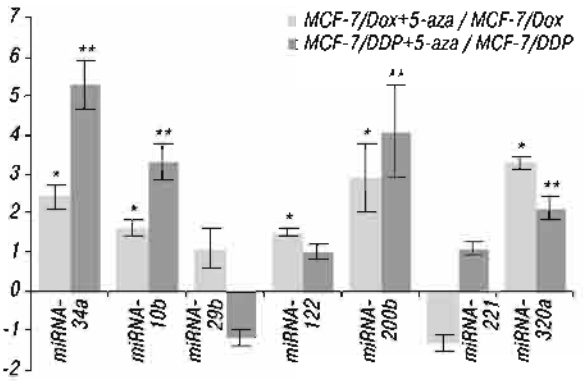

Fig. 2. miRNAs expression patterns after 5-aza treatment in resistant MCF-7 cell sublines compared with the control cell cultures; *p < 0.05 compared with the control MCF-7/Dox cell culture; **p < 0.05 compared with the control MCF-7/DDP cell culture. Average ± SD values are shown

The results of the present work show that preliminary cultivation of MCF-7 cells with 5-aza resulted in increase of cytotoxic effects of DDP and Dox (Table 2). In particular, the IC50 of Dox decreased by 2, 4 and 3 fold for cell lines MCF-7/S, MCF-7/Dox and MCF-7 /DDP cells, respectively.

IC50 of DDP in studied cultures decreased by 3, 2 and 1.5 fold, respectively.

Such modulating effect of 5-aza on cytotoxic effects of Dox can be explained by promoter demethylation of *MDR1* gene, which is responsible in elimination of Dox from cells. There are twelve CpG-islands in the *MDR1* gene promoter, 5 of which remain methylated in transformed cells. Increased expression of this gene is usually caused by its amplification. It is proposed that demethylation facilitates binding of regulatory elements to the promoter, inhibiting the transcription of this gene [26].

Also, increased miRNA-320a is involved in *MDR1* silencing, as well as miRNA-34a does [1, 17, 30].

It was proposed earlier, that increase of sensitivity to DDP could be associated with the following mechanisms — 5-aza causes DNA demethylation, and this leads to changes in expression of genes responsible for the transport of DDP into the cell. DDP is associated with GG, AG and GNG motifs, forming intra-chain DNA adducts and extra- and intrastrand G-G DNA crosslinks. Demethylation of CpG-rich areas increases their vulnerability to DDP and, thereby increases sensitivity [19, 26, 31].

Table 2. Increased sensitivity of BC cells to anticancer drugs after treatment with 5-aza

	IC50, μM					
	MCF-7/S		MCF-7/DOX		MCF-7/DDP	
	Dox	DDP	Dox	DDP	Dox	DDP
Control	4.1 ± 0.3	15.3 ± 1.3	23.3 ± 2.1	16.0 ± 1.0	12.4 ± 1.2	93.3 ± 7.0
10 days with 5-aza	3.5 ± 0.2*	10.5 ± 1.9*	13.7 ± 1.3*	11.9 ± 0.8*	10.2 ± 1.2*	87.4 ± 3.0*
20 days with 5-aza	2.9 ± 0.7*	7.2 ± 1.4*	6.3 ± 1.1*	9.7 ±1.5*	6.9 = 0.9*	55.9 ± 2.1*
30 days with 5-aza	2.4 ± 0.3*	5.4 ± 0.3*	5.2 ± 0.2*	4.4 ± 2.4*	4.6 ± 0.2*	45.2 ± 6.3*

Note: *p < 0.05 compared with the control cell culture. Average ± SD values are shown.

Another mechanism to restore the sensitivity to DDP is increased miRNA-200b expression. This miRNA modulates iron metabolism, thus, enhancing oxidative stress in cancer cells [19, 32].

In conclusion, we have shown that miRNA expression was increased in BC cells upon 5-aza treatment, due to promoter DNA demethylation. At such conditions restoration of expression of miRNA-10b, -34a, -200b, and -320a was observed in MCF-7 sublines resistant to Dox and DDP. Importantly, this increased sensitivity of prior resistant cells to treatment with anti-cancer drugs.

We may speculate that this phenomenon is due to the enhanced epigenetic control of miRNA expression upon treatment with 5-aza. The obtained data create the basis for further *in vivo* investigations.

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