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ORIGINAL CONTRIBUTION

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MODIFYING EFFECTS OF 5-AZACYTIDINE ON METAL-CONTAINING PROTEINS PROFILE IN GUERIN CARCINOMA WITH DIFFERENT SENSITIVITY TO CYTOSTATICS

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Aim*: To assess the influence of the treatment with 5-azacytidine (5-aza) on the profile of metal-containing proteins and factors of their regulation in Guerin carcinoma cells *in vivo*. *Materials and Methods:* The study was conducted on Wistar rats transplanted with wild-type Guerin carcinoma (Guerin/WT) and its strains resistant to cisplatin (Guerin/CP) or doxorubicin (Guerin/Dox). Animals were distributed in 6 groups treated with 5-aza and control animals without treatment. 5-Aza was injected by i.v. route (1 injection in 4 days at a dose of 2 mg/kg starting from the 4th day after tumor transplantation, 4 injections in total). Ferritin levels in blood serum and tumor tissue were measured by ELISA, transferrin and free iron complexes — by low-temperature EPR, miRNA-200b, -133a and -320a levels and promoter methylation — by real-time quantitative reverse transcription polymerase chain reaction. *Results*: The study has shown that 5-aza treatment caused demethylation of promoter regions of *fth1* and *tfr1* genes in all studied Guerin carcinoma strains. 5-Aza treatment resulted in a significant decrease of ferritin levels in tumor tissue (by 32.1% in Guerin/WT strain, by 29.8% in Guerin/Dox and by 69.1% in Guerin/CP). These events were accompanied by 3.5-fold and 2-fold increase of free iron complexes levels in tumor tissue of doxorubicin and cisplatin resistant strains, respectively. Also, 5-aza treatment resulted in significantly elevated levels of miR-200b, -133a, 320a expression in tumor tissue. After 5-aza treatment, ferritin levels in blood serum of animals with Guerin/Dox were increased by 23.9%, while in Guerin/Wt and Guerin/CP they were decreased by 17 and 16%, respectively. *Conclusion*: Alterations of epigenetic regulation upon *in vivo* treatment with 5-aza change the levels of metal-containing proteins due to DNA demethylation and altered miRNA expression profiles in Guerin carcinoma cells.

***Key Words*: metal-containing proteins, 5-azacytidine, Guerin carcinoma, resistance, methylation, microRNA.**

Despite recent advances in understanding tumor nature, many questions remain unanswered. One of them is the possibility of exogenous correction of certain tumor features. Solving this problem would give us radically new approaches in cancer therapy, which would make tumors more sensitive to cytostatic drugs and allow to decrease toxic side effects [1].

One of the most promising targets, which could significantly affect tumor features, is iron metabolism because this element is a vital cofactor for many important cellular processes (energy synthesis, transcription, cell division, oxidation reactions etc.) [2]. During last few years it has been shown that many features of cancer cells *in vitro* and tumors *in vivo* are associated with different aspects of iron metabolism. In our previous works we already described the changes in this process, in particular, altered expression of different metal-containing proteins in tumor cells with different degree of malignancy and different sensitivity to cisplatin and doxorubicin [3]. Also, we revealed that Walker-256 carcinosarcoma showed gradual changes in the levels of different metal-containing proteins at different stages of the development of resistance to doxorubicin. Those changes were observed both in tumor tissue and in blood serum of the host organism [4].

Another problem is the search for useful mechanisms to correct mentioned changes in iron metabolism and the levels of metal-containing proteins [5]. One of the promising approaches is related to epigenetic mechanisms of regulation of protein expression, such as changes in DNA methylation patterns and miRNA expression profiles [6].

In 1974 Czechoslovakian scientists synthesized 5-aza-2'deoxycytidine (5-azacytidine) which was a new potential chemotherapeutic drug [7]. Later other scientists discovered that mechanisms of cytotoxic action of high dose 5-azacytidine (5-aza) were based on its incorporation into DNA and RNA, resulting in breaks in nucleic acid chains and cell apoptosis, while low doses of this compound inhibited DNA methyltransferase activity and caused DNA hypomethylation. This demethylation activity could result in altered profile of metal-containing proteins and changed tumor phenotype [8].

So, the aim of our study was to assess the influence of the treatment with 5-aza on the profile of metal-containing proteins and factors of their regulation in Guerin carcinoma cells *in vivo*.

MATERIALS AND METHODS

Experimental animals. Studies were carried out on 60 Wistar female rats with subcutaneously transplanted Guerin carcinoma strains, wild type (WT) or resistant to cisplatin (Guerin/CP) and doxorubicin

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Abbreviations used: 5-aza – 5-azacytidine; CP – cisplatin; Dox – doxorubicin; EPR – electron-paramagnetic resonance; FTH – ferritin heavy chains; MSP – methylation-specific PCR; PCR – polymerase chain reaction; qRT-PCR – real-time quantitative reverse transcription PCR; TFR – transferrin receptor.

(Guerin/Dox) ($2 \cdot 10^6$ cells per animal). These tumor strains were obtained from National Bank of Cell Lines and Transplanted Tumors of R.E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology, National Academy of Sciences of Ukraine (Kyiv, Ukraine). The use and care of the experimental animals have been performed in accordance with the standard international rules of biologic ethics and was approved by Institutional Animal Care and Use Committee.

Animals were distributed in 6 groups (10 animals per group): 1) Guerin/WT, control; 2) Guerin/WT treated with 5-aza; 3) Guerin/CP, control; 4) Guerin/CP treated with 5-aza; 5) Guerin/Dox, control; 4) Guerin/Dox treated with 5-aza.

10 animals from each group received 5-aza (Gedeon-Richter, Czech Republic) treatment: 1 injection each 4 days i/v at a dose of 2 mg/kg starting from the 4th day after tumor transplantation, 4 injections in total. All of the studied parameters were measured on the 20th day after tumor transplantation.

ELISA. Ferritin levels in blood serum and tumor tissue were measured by automatic biochemical and immunoenzyme analyzer Chem Well 2900 (USA) using ferritin ELISA kit (Uscer, China) according to the instruction of the manufacturer. Blood serum samples were collected and tumor homogenate was prepared and stored at −20 °C.

Low-temperature electron-paramagnetic resonance (EPR). Transferrin levels in blood serum and tumor tissue were measured using EPR method using computerized spectrometer R-1307 (Russia) at 77 K, EDTA sodium salt was used as anticoagulant. Stripe width was 1525 G, frequency — 9.15 GHz, microwave power — 40 mW, amplitude modulation — 10.0 G, frequency modulation — 100 kHz. g-Factor was calculated using standard formula:

$$g = h\nu/\beta H,$$

where h is Plank's constant, ν — frequency, β — Bohr's magneton, H — external magnetic field in resonance.

Apoptosis and necrosis rates were estimated using classical Annexin V/PI method as previously described [5] with the use of BeckmanCoulter EPICS® XL Flow cytofluorimeter.

Total DNA isolation. Total DNA extraction was performed with "DNA-Sorb B" DNA Isolation Kit (Amplisens, Russia) according to the manual, provided by manufacturer.

Methylation-specific polymerase chain reaction (PCR) (MSP). Bisulfite conversion involves the deamination of unmodified cytosine residues to uracil under the influence of hydrosulfite ion from water solution of sodium bisulfite. Such treatment doesn't affect 5-mC and in further amplification uracils are amplified as thymines, whereas 5-mC residues get amplified as cytosines. Bisulfite conversion was performed using EZ DNA Methylation Gold-Kit (Zymo Research, USA) according to manufacturer's protocol. Aliquots of bisulfite-modified DNA were stored at −20 °C and were used for MSP. MSP was performed using the standard protocols; primer sequences are available in Table 1. TSH2B and GAPDH were used as reference control genes [9].

Table 1. Primer sequences, used in MSP

Gene	Primer sequence	Size of resulting product
FTH1_M	F: 5' – cga ggg ttt tta gcg gtc – 3' R: 5' – atc tct tat aac cgc gtc gac – 3'	128 bp
FTH1_U	F: 5' – gtg agg gtt ttt agt ggtt – 3' R: 5' – aat ctc tta taa cca cat caa c – 3'	128 bp
TFR1_M	F: 5' – gta gtt ggg att ata ggc gc – 3' R: 5' – taa tta cca aac gcg ata act c-3'	180 bp
TFR1_U	F: 5' – tga gta gtt ggg att ata ggt gt – 3' R: 5' – taa tta cca aac aca ata act cac – 3'	180 bp

Notes: FTH1_M – ferritin heavy chains, methylated promoter; FTH1_U – ferritin heavy chains, unmethylated promoter; TFR1_M – transferrin receptor 1, methylated promoter; TFR1_U – transferrin receptor 1, unmethylated promoter.

PCR products were analyzed by agarose gel electrophoresis in 1.2% agarose "Low EEO, Type 1-A" (Sigma, USA). After electrophoresis the results was visualized by ethidium bromide, photographed under UV light and evaluated by a computer program TotalLab v2.01.

Total RNA isolation. Total RNA extraction was performed with "Ribozol" RNA Isolation Kit (Amplisens, Russia). RNA concentration was measured with NanoDrop 2000c Spectrophotometer (Thermo Scientific, USA). Purity of isolated RNA was controlled by 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 PCR (qRT-PCR). Reaction mix for reverse transcription was prepared according to manufacturer's protocol. Reverse transcription was performed with thermal cycler "Tertsik" ("DNA Tehnologiya", Russian Federation). 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 [8]. 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:

$$Fold\ change = 2{-}\Delta\Delta Ct\ [8],$$

where ΔCt (target — control) is equal to the difference between threshold cycles for miRNA (target) and the threshold cycle for RNU48 (control):

$$\Delta Ct\,(target - control) = Ct\,(target) - Ct(control);$$
$$\Delta\Delta Ct = \Delta Ct\,(experiment) - \Delta Ct\,(control).$$

Statistical analysis. Experimental data were analyzed 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

It is known that demethylating agents, such as 5-aza, enhance the expression of oncosuppressive genes [10]. In addition to affecting methylation status of promoter CpG islands, 5-aza targets are transcription factors that are involved in the cell cycle regulation. In particular, c-Myc activates expression of the transferrin receptor 1 (TfR1), ferritin and iron-regulated protein 2 (IRP2). These proteins are important regulators of iron metabolism in the cell, and are critical for proliferation and division of tumor cells [11].

Web resources (https://www.ebi.ac.uk/Tools/seqstats/emboss_cpgplot and https://genome.ucsc.edu) were used to analyze DNA segments at a distance of 1.5 kb in up- and downstream regions from the transcription start sites. We found regulatory CpG-islands in promoter regions of transferrin receptor 1 (*tfr1*) and ferritin heavy chains (*fth1*) genes, as well as miRNA-133a, -200b, and -320a.

Thus, we decided to investigate the changes in methylation status of CpG-islands in *tfr1* and *fth1* gene promoter areas in Guerin carcinoma strains, wild-type and resistant to doxorubicin and cisplatin, after the session of 5-aza-based therapy. We established that the development of resistance to doxorubicin and cisplatin is accompanied by hypomethylation of studied CpG areas (Table 2). After 5-aza treatment, levels of the *fth1* and *tfr1* promoter methylation in all strains were almost identical pointing on their demethylation. The level of promoter methylation of transferrin receptor 1 gene in the sensitive and resistant to doxorubicin strains after 5-aza treatment were identical, while in cisplatin-resistant strain it was two times higher (see Table 2).

However, the level of ferritin protein in the tumor tissue under the influence of 5-aza was reduced in all strains (Fig. 1), indicating that additional regulatory mechanisms are involved in response to genome methylation changes.

Another epigenetic mechanism, responsible for protein expression regulation is RNA interference, in which miRNAs play the major role. miRNAs are small noncoding RNAs, which regulate the expression of proteins at post-transcriptional level. For example, the targets of oncosuppressor miRNA-200b and -133a are mRNA of heavy (FTH1) and light (FTL) chains of ferritin molecule. Together with CpG methylation of these genes, miRNA-200b and -133a regulate the presence of ferritin in the cells [12].

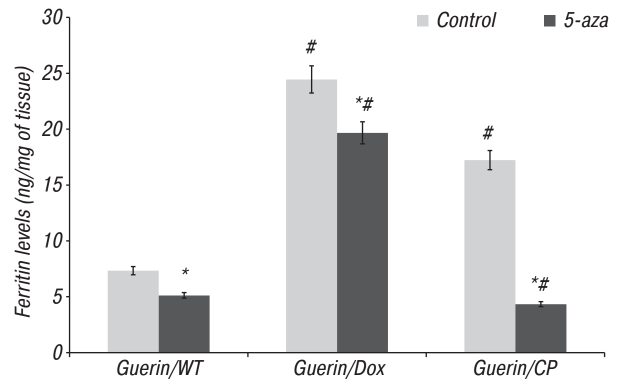

Fig. 1. Changes of ferritin levels in tumor tissue of Guerin carcinoma sensitive and resistant to cytostatics after 5-aza treatment. *p < 0.05 compared with control group; #p < 0.05 compared with control WT strain

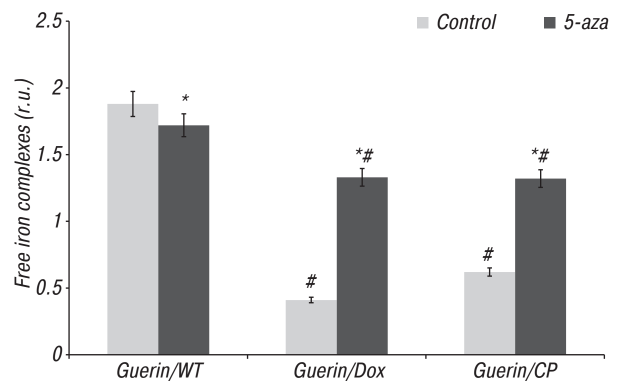

Fig. 2. Levels of free iron complexes in Guerin carcinoma tissue after 5-aza treatment. *p < 0.05 compared with control group; #p < 0.05 compared with control WT strain

It was recently shown (on heme binding protein DGCR8) that the changes in iron balance in cells play a key role in the maturation of different miRNAs [13]. Iron-bound transferrin binds to transferrin receptors 1 and 2 (TfR1, 2) on a cell surface and enters the cell via endocytosis. Then divalent metal transporter 1 (DMT1) releases the iron into the cytoplasm, forming the "labile iron pool", which can be stored in ferritin or used by mitochondria to synthesize heme. Heme is critical for the maturation of pre-miRNA transcripts because it binds to the processing complex, which consists of DGCR8 and Drosha proteins. Another way of iron-dependent regulation is pre-miRNAs export from the nucleus to the cytoplasm, where,

Table 2. Changes of *fth1* and *tfr1* promoter methylation levels in Guerin carcinoma cells after 5-aza treatment

Genes	Level of promoter methylation (r. u.)					
	Guerin/WT		Guerin/Dox		Guerin/CP	
	Control	+ 5-aza	Control	+ 5-aza	Control	+ 5-aza
fth1	7.15 ± 0.24	2.40 ± 0.31*	4.50 ± 0.20#	2.30 ± 0.10*#	5.34 ± 0.30#	2.70 ± 0.60*#
tfr1	1.63 ± 0.01	1.27 ± 0.02*	2.25 ± 0.18#	1.21 ± 0.12*#	3.50 ± 0.10#	2.60 ± 0.11*#

Note: *p < 0.05 compared with control group; #p < 0.05 compared with control WT strain.

Table 3. Changes in expression of miRNA-133a, -200b and -320a in Guerin carcinoma tumor tissue after 5-aza treatment

ΔCt	Guerin carcinoma strain					
	WT		Guerin/Dox		Guerin/CP	
	Control	+ 5-aza	Control	+ 5-aza	Control	+ 5-aza
miR-133a	0.81 ± 0.01	1.72 ± 0.02*	0.41 ± 0.02#	1.33 ± 0.01*#	0.62 ± 0.01#	1.32 ± 0.01*#
miR-200b	0.70 ± 1.25	4.60 ± 0.61*	0.42 ± 0.05#	0.60 ± 0.01*	0.35 ± 0.25#	9.70 ± 0.15*#
miR-320a	0.29 ± 0.19	0.46 ± 0.08*	0.05 ± 0.04#	0.35 ±0.03*	0.11 ± 0.03#	0.39 ± 0.01*

Note: *p < 0.05 compared with control group; #p < 0.05 compared with control WT strain.

depending on iron levels, C-domain-binding protein PCBP2 forms active dimers. Dimerized PCBP2 binds DICER resulting in miRNA maturation. Increase of iron levels in a cell leads to PCBP2 monomerization and its transition into inactive form [14].

Measurement of free iron complexes by EPR spectroscopy (Fig. 2) showed that 5-aza treatment caused no changes in Guerin/WT cells. On the other hand, levels of free iron complexes in both resistant strains significantly elevated after 5-aza treatment and became close to those of the sensitive strain.

Next, we have studied the effects of 5-aza on the levels of miRNA, which regulate different metal-containing proteins. We found that 5-aza treatment caused the changes in the levels of miRNA-133a, -200b and 320a in tumor cells of all studied Guerin carcinoma strains. Changes of the expression in miR-133a after demethylating agent exposition had unidirectional nature in sensitive and resistant Guerin carcinoma cells (Table 3).

We determined that 5-aza treatment resulted in the increase of miR-133a and -320a levels in all three strains. Levels of miR-200b also increased in all studied strains, but were the most pronounced in Guerin/CP cells. Obtained data on miRNA-133a and -200b expression were in good accordance with the data on ferritin levels in tumor tissue, mentioned above. So, despite hypomethylation of its promoter, *fth1* expression is silenced by miRNAs on the post-transctriptional level.

It is known that *tfr1* and *mdr1* mRNAs are direct targets of miRNA-320a. Overexpression of both proteins is observed in many cancer cells, resistant to different cytostatics. So, we speculate that the increase of microRNA-320a expression caused by 5-aza, could sensitize malignant cells to anticancer drugs [15].

Due to constant binding of DNMT enzymes, 5-aza causes apoptotic effect on poorly differentiated stem cells, but the exact mechanism of this influence is still unclear [16]. We assessed the apoptotic and necrotic rates of Guerin carcinoma cells, and it was found that in Guerin/WT the treatment with 5-aza resulted in the increased number of cells in a state of necrosis and apoptosis — by 13.7 and 49%, respectively, compared to untreated control. In Guerin/CP and Guerin/Dox cells under the influence of 5-aza, the number of cells in a state of necrosis did not change, and the number of cells in a state of apoptosis increased twice (Table 4).

So, we can conclude that 5-aza treatment resulted in unidirectional changes in all studied Guerin carcinoma strains. Particularly, we observed hypomethylation of *fth1* and *tfr1* promoters, and, as a result, elevation of free iron levels in tumor cells. This caused significant increase of expression of miRNAs involved in regulation of iron levels, resulting in silencing of ferritin expression and the loss of resistance to cytostatics.

These changes in tumor behavior were confirmed on organism level. After 5-aza treatment, in blood serum of animals with Guerin/Dox ferritin levels were increased by 23.9%, while in Guerin/WT and Guerin/CP — decreased by 17 and 16%, respectively. Also, after 5-aza treatment we observed lowering of ceruloplasmin activity (see Table 4). These results indicate that organism's iron stores are depleted (in accordance with the data on increase of free iron in tumor tissue). Decrease of ceruloplasmin activity in blood serum (as we showed in our previous studies) is a non-tumor marker of tumor sensitivity to chemotherapy.

So, we can conclude that of 5-aza can be used as an exogenous modifier of the content of metal-containing proteins both in tumor and in an organism. Moreover, 5-aza was capable to change some features of drug-resistant tumors, making them more sensitive to cytostatics because of *mdr1* deregulation and decrease of ceruloplasmin activity in blood serum.

Our results demonstrated that 5-aza could be considered as a promising agent, which can be used in low doses in combination with convenient chemotherapy (especially cisplatin and doxorubicin) to increase antitumor effects.

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Table 4. Indices of metal-containing proteins in blood serum of animals with Guerin carcinoma after 5-aza treatment

Proteins	Guerin carcinoma strain					
	WT		Guerin/Dox		Guerin/CP	
	Control	+ 5-aza	Control	+ 5-aza	Control	+ 5-aza
Ferritin, ng/ml	13.43 ± 1.19	11.20 ± 1.05#	12.11 ± 1.23	15.90 ± 1.13*	12.50 ± 1.21	10.50 ± 1.10*#
Transferrin, r. u.	0.17 ± 0.02	0.23 ± 0.01#	0.27 ± 0.01	0.30 ± 0.01*#	0.22 ± 0.09	0.34 ± 0.01*
Ceruloplasmin, r. u.	5.12 ± 0.11	4.10 ± 0.23#	2.30 ± 0.01#	1.80 ± 0.03*	2.60 ± 0.01*#	2.0 ± 0.02*#

Note: *$p < 0.05$ compared with untreated strain; #$p < 0.05$ compared with untreated sensitive strain.

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