ANTICANCER RESEARCH *40*: 795-805 (2020)
doi:10.21873/anticanres.14011

Approbation of the Cancer Treatment Approach Based on the Eradication of TAMRA+ Cancer Stem Cells in a Model of Murine Cyclophosphamide Resistant Lymphosarcoma

POLINA E. KISARETOVA[1*], SVETLANA S. KIRIKOVICH[1*], GENRICH S. RITTER[1,2], YAROSLAV R. EFREMOV[1,2], OLEG S. TARANOV[3], TATYANA D. DUBATOLOVA[4], ANASTASIA S. PROSKURINA[1], EKATERINA A. POTTER[1], EVGENIA V. DOLGOVA[1], SERGEY V. SIDOROV[2,5], ALEKSANDR A. OSTANIN[6], ELENA R. CHERNYCH[6] and SERGEY S. BOGACHEV[1]

[1]*Institute of Cytology and Genetics, Siberian Branch of the Russian Academy of Sciences, Novosibirsk, Russia;*
[2]*Novosibirsk State University, Novosibirsk, Russia;*
[3]*State Research Center of Virology and Biotechnology "VECTOR", Novosibirsk, Russia;*
[4]*Institute of Molecular and Cellular Biology, Siberian Branch of the Russian Academy of Sciences, Novosibirsk, Russia;*
[5]*Oncology Department of Municipal Hospital No 1, Novosibirsk, Russia;*
[6]*Research Institute of Fundamental and Clinical Immunology, Novosibirsk, Russia*

Abstract. *Background/Aim: We previously have described the "3+1" tumors cure approach consisting of individual time schedule of cyclophosphamide and dsDNA preparation administrations. The aim of the study was to adapt the "3+1" approach based on eradication of cancer stem cells to the model of murine ascitic cyclophosphamide-resistant lymphosarcoma (RLS). Materials and Methods: Adaptation of the "3+1" approach includes the identification of the timing to disrupt the tumorigenic potential of a certain tumor. Results: The proposed therapeutic scheme allowed complete reduction of primary RLS ascites in experimental animals. However, reduction of primary ascites due to the complementary action of cyclophosphamide and dsDNA was inevitably followed by the development of a secondary one, most likely arising from a solid carcinomatous formation in the peritoneal wall. Conclusion: The "3+1" approach resulted in the elimination of cancer stem cells, and, as a consequence, in the complete reduction of RLS ascites.*

*These Authors contributed equally to this work.

Correspondence to: Sergey S. Bogachev, Institute of Cytology and Genetics, Siberian Branch of Russian Academy of Sciences, 10 Lavrentyev ave, Novosibirsk 630090, Russia. Tel: +7 3833634963, Fax: +7 3833331278, e-mail: labmolbiol@mail.ru ORCID: https://orcid.org/0000-0002-2019-9382

Key Words: RLS lymphosarcoma, cyclophosphamide, double-stranded DNA, secondary ascites, cancer stem cells eradication.

Malignant neoplasms rank second in the world after cardiovascular disorders in overall mortality rates. Recent studies indicate a much higher complexity of the disease than previously thought. The main characteristic of this complexity determining the unpredictable response of neoplasms to a variety of therapeutic procedures and the difficulties in their cure is the heterogeneity of malignant cells that include a subpopulation of almost "indestructible" cells, which possess an incredible survival and proliferative potency, and is designated as tumor-initiating stem cells or cancer stem cells (CSCs) (1, 2).

The main hallmarks of cancer stem cells are their capabilities of (1) self-renewal in through unlimited replicative cycles, (2) producing a progeny of committed cells with high proliferative activity, but incapable of inducing a new tumor, and (3) retaining a potency to induce new tumors with similar histological properties in a series of transplantations (3). CSCs possess a number of features allowing their dominance in co-existence with organism: self-sufficiency in proliferative stimuli (4), reduced sensitivity to anti-proliferative ones (5), immortalization (6, 7), dedifferentiation (8), genomic instability (9), increased efflux and metabolism of xenobiotics (10, 11), reversed "Warburg effect" (12, 13), suppression of pro-apoptotic signals (14), as well as stimulation of pathways causing apoptosis evasion (15). Another essential for disease development property of CSCs is their migratory capacity, allowing exit into the bloodstream, and formation of new growth foci in distant organs (16-20). Thus, the aforementioned properties of CSCs are such that they create

fundamental obstacles in developing therapeutic approaches, which could override their resilience (15), determined by their resistance to chemo- and radiotherapy (9, 21).

Both detection and elimination of CSCs from a tumor are key outcomes for curing malignant neoplasms. In this regard, the priority of identifying CSCs among the bulk of tumor cells is inevitably followed by the question of how to kill these cells in their native environment. To develop approaches aimed at precise destruction of CSCs, their presence in a variety of tumors, as well as their properties, are being investigated to identify specific therapeutic targets (22-24). These targets, however, are neither universal for all types of cancer nor even stably specific for the same one (25). Thus, the development of new and adequate therapeutic approaches and agents precisely aimed at elimination of CSCs remains an urgent task; their implementation will be a great leap in curing malignant neoplasms.

Over the past 20 years, we have found and described a new and previously unknown phenomenon of general biological significance – the capability of poorly differentiated cells, including cancer stem cells, to internalize fragments of extracellular double stranded DNA (dsDNA) without any artificial manipulation. DsDNA probe labeled with fluorescent dye provides a universal and, at the same time, unique marker of stem cells of various origin, including CSCs (26-34). We have also established that dsDNA fragments internalized into CSCs during the process of repairing inter-chain crosslinks induced by exposure to cyclophosphamide interfere in this process, completely disrupting the grafting potential of these cells. The loss of grafting potential was found to be due to the elimination of CSCs from the bulk of transplanted tumor cells. In addition, we have determined the timing of cyclophosphamide treatments required for synchronizing these cells in a certain phase of the cell cycle when they are extremely sensitive to dsDNA (29-34). Based on these findings, the new approach named "3+1" or "Karanakhan" (sanskrit – "killing the source"). This approach allows direct eradication of CSCs, which are the source of tumor onset and development, and implies the following procedures: 1. Detection of poorly differentiated CSCs in a tumor by their ability of internalizing TAMRA-labeled dsDNA probe; 2. Identification of the time profile for inter-chain crosslinks repair cycle after exposure to a crosslinking cytostatic agent; 3. Synchronization of proliferating cells, including CSCs, in the late $S/G_2/M$ phase of the cell cycle by triple exposure to a cytostatic in accordance to determined DNA repair cycle length, followed by determination of the time when these cells synchronously exit this phase and accumulate in G_1, becoming vulnerable to the terminal treatment (30); 4. Administration of the composite dsDNA-based preparation at the point demarcating the phases of nucleotide excision repair and homologous recombination. One of the components interferes with the nucleotide excision repair, while the other with the homologous recombination, causing CSCs either to completely lose their ability of surviving the "therapeutic strike", or, at least, to forfeit their tumor-initiating properties. Moreover, this therapeutic strategy will result in rapid large-scale lysis of the bulk of committed tumor cells, especially those actively proliferating.

Currently, this approach has been successfully tested, providing a good therapeutic effect in experimental murine carcinoma Krebs-2 in both ascitic and solid forms (30, 31), human glioblastoma U87 (33), and murine hepatocarcinoma G29 (32).

In this study, the murine ascitic cyclophosphamide (CP) resistant lymphosarcoma RLS was used as a model. This neoplasm was obtained from the murine lymphosarcoma LS susceptible to cyclophosphamide as a result of multiple low-dose (20 mg/kg) administrations of CP followed by transplantation (35). Cells of the ascitic lymphosarcoma RLS are featured with the lowered expression of *p53* tumor suppressor gene and initially elevated expression of anti-apoptotic *Bcl-2* gene (35), which is subsequently lost after multiple *in vivo* passages (36). Additionally, RLS is characterized by overexpression of genes belonging to ABC superfamily of transmembrane transporters (*Mdr1a/b*) (35), allowing effective efflux of CP metabolites and preventing their intracellular accumulation in quantities essential for apoptosis initiation (37). *In vitro*, 10% of RLS cells have been reported to display multiple drug resistance (35). These cells could be presumed to be either the subpopulation of cancer stem cells themselves, or, at least, a subpopulation of them.

Preliminary data indicated the presence of approximately 1% of TAMRA-positive cells in intact RLS ascites. It is possible that these cells (as well as their closest progeny, already incapable of internalizing TAMRA-labeled DNA probe) constitute the subpopulation of the least committed cells with multiple drug resistance phenotype. Identification of TAMRA+ cells in the total population of RLS lymphosarcoma cells implied the possibility of using this cell line as a model for further validating the novel "3+1" approach aimed at direct elimination of CSCs.

The current report is a part of the large-scale research aimed at adapting the recently developed cancer treatment approach to a variety of experimental cancer models (Krebs-2, murine hepatocarcinoma G-29, human glioblastoma U-87 *etc.*).

Materials and Methods

Breeding experimental animals. Inbred CBA/Lac mice aged 2-3 months were provided by the vivarium of the Institute of Cytology and Genetics SBRAS. Animals were kept in plastic cages (10 animals per each) with free and unlimited access to food and water. Animals were fed with granulated food PK120-1 (Laboratorsnab, Moscow, Russia).

Tumor model. The cyclophosphamide resistant murine lymphosarcoma RLS (38, 39) obtained from the repository of Institute of Cytology and Genetics SBRAS was used as a model. For grafting, cells were sampled from mice with 10-day ascites, diluted with physiological saline solution and inoculated into animals at the amount of 10^6 per mouse to produce the ascitic form of the tumor.

Administration of preparations of cyclophosphamide/mitomycin C and exogenous DNA. CP was administered into mice intraperitoneally at the dose of 100 mg/kg of body weight. The DNAmix® preparation (31) was administered intraperitoneally at doses of 0.5-1 mg per injection. For *in vitro* treatments, 1-10 μg/ml mitomycin C instead of CP was.

In vivo therapy with in vitro pretreatment. Prior to transplanting into mice, RLS lymphosarcoma cells were subjected to a single *in vitro* exposure to mitomycin C (10 μg/ml) and DNA (50 μg) to presumably prevent carcinomatosis. Then, 7 groups of mice (5 animals each) were engrafted with 10^6 of these pretreated cells per mouse. CP (100 mg/kg) was administered three times with an interval of 36 h. The terminal CP treatment (100 mg/kg) was conducted on day 5, 9 or 12. Eighteen h after each CP administration, mice were administered with 0.3 mg of DNA preparation per mouse.

Labeling DNA with TAMRA (tetramethylrhodamine) fluorescent dye. TAMRA-labeled Alu fragment was produced by PCR conducted in 50 μl containing 1×Taq-buffer (Medigen), 2 mM $MgCl_2$, 0.2 mM dCTP, 0.2 mM dGTP, 0.2 mM dATP, 0.16 mM dTTP, 50 pmol pr M13 for, 50 pmol pr M13 rev, 0.4 μg Alu I in PBS, 5 u Taq-polymerase, 0.05 mM dUTP-TAMRA. The following cycling program was used: 1 cycle: 95˚C – 5 min; 33 cycles: 95˚C – 40 s; 56˚C – 40 s; 72˚C – 40 s; 1 cycle: 72˚C – 5 min; The obtained TAMRA-Alu DNA was stored at 4˚C.

Analysis of TAMRA-labeled DNA internalization into RLS ascites cells. RLS ascites cells were sampled from a mouse, sedimented at 400×g for 5 min at 4˚C, washed once with RPMI-1640 medium, and counted in a Goryaev chamber. About 10^6 cells were incubated in 200 μl of serum-free RPMI-1640 with 200 ng of TAMRA-labeled DNA for 1 h at room temperature in the dark. After incubation, cells were sedimented, washed with a small volume of medium, and resuspended in the final volume of the medium. Further, the number of cells with internalized TAMRA-DNA was estimated on FACSAria III (BD Biosciences, San Jose, CA, USA) and/or Axioskop 2 Plus (Zeiss, Jena, Germany) with AxioVision software. For microscopic analysis, 50-100×10^3 cells were applied on a glass slide using cytospin (1,000 rpm for 1 min), and following addition of ~10 μl of DABCO Antifade they covered with a coverslip and analyzed. The percentage of TAMRA+ cells was estimated relatively to a control sample incubated in medium without TAMRA-DNA.

Detection of the length of CP-induced interchain crosslinks (ICLs) repair cycle in RLS ascitic lymphosarcoma cells by the "comet tails" method. After appropriate treatments, RLS ascites cells were sampled from animals, washed with RPMI-1640 and sedimented at 400×*g* for 5 min. After sedimentation, cells were resuspended in RPMI-1640 and counted in a Goryaev chamber. Approximately 7×10^3 cells were sampled and diluted with medium to the final volume of 35 μl. Then, an equal volume of 2% low-melting agarose was added and 35 μl of the obtained suspension were added to special wells (1 mm×3 mm×9 mm size) and left for 10 min at 4˚C. The obtained agarose blocks were placed in 500 μl EDTA (0,5 M; pH=8.0) and stored at 4˚C. Prior to electrophoresis, the blocks were lysed in 1% sodium sarcosyl for 1h at 37˚C. Electrophoresis was carried out at 36 V/cm^2 for 20 min. Electrophoresized blocks were then placed on a glass slide (3 blocks per slide) and dried overnight at 37˚C. The dried blocks were further washed with distilled water for 30 min and analyzed on a fluorescence microscope Axioskop 2 Plus (Zeiss) with AxioVision software. The length of "comet tails", or tail moment (TM), was estimated using CASP software.

Estimation of the RLS cells synchronization time after successive therapeutic exposures to CP. Animals with well developed ascites were administered three times with 100 mg/kg of CP. Ascites cells from five mice were sampled on day 1 (as a control point), and then every 24 h during 5-12 days of the experiment. A fraction of the sampled cells was fixed with 50% methanol. Fixed cells were incubated with RNase and propidium iodide for 30 min at 37˚C, and analyzed on FACSAria to estimate the cell cycle profile. The remaining live cells were incubated with TAMRA-labeled DNA probe for microscopic estimation of the number of DNA-internalizing cells.

Statistical analysis. Statistical analysis of the data was performed using Microsoft Excel and Statistica 10 package. The tail moment values were tested for Gaussian distribution by the Shapiro–Wilk test (testing the identity of the observed distribution and the theoretically expected Gaussian one) and for differences confidence by the Student's *t*-test using Statistica 10 software package. Survival time in groups of animals was compared (relatively to the control) using non-parametric statistics with the Mann–Whitney *U*-test from Statistica 10 package.

Results

Internalization of TAMRA-labeled DNA probe by RLS murine ascitic lymphosarcoma cells. Since the main target of the proposed therapeutic approach were CSCs, their detection in the bulk of RLS ascitic cells was the first and main objective. Fluorescence microscopic (Figure 1A) and FACS (Figure 1B) assays proved the presence of TAMRA-positive cells in RLS ascites. TAMRA+ CSCs were shown to constitute approximately 1% of the total RLS ascitic cells.

The length of ICL repair cycle in RLS ascitic lymphosarcoma cells, detected by the "comet tails" method after a single CP administration. A 6-day ascites (the stage when a biopsy sample can be easily taken) was used. Ascites-bearing mouse was administered once with 100 mg/kg CP. Ascitic cells were sampled every 6 h after CP administration till the final sampling at 48 h. DNA double-strand breaks in ascites cells were detected by the "comet tails" method. The start of the ICLs repair is associated with appearance and accumulation of double-strand breaks, which indicate the nucleotide excision repair phase. During the second, homologous recombination phase of the repair process, double-strand breaks are being repaired and gradually disappear. Complete disappearance of double-strand breaks indicates the end of the repair process. The total length

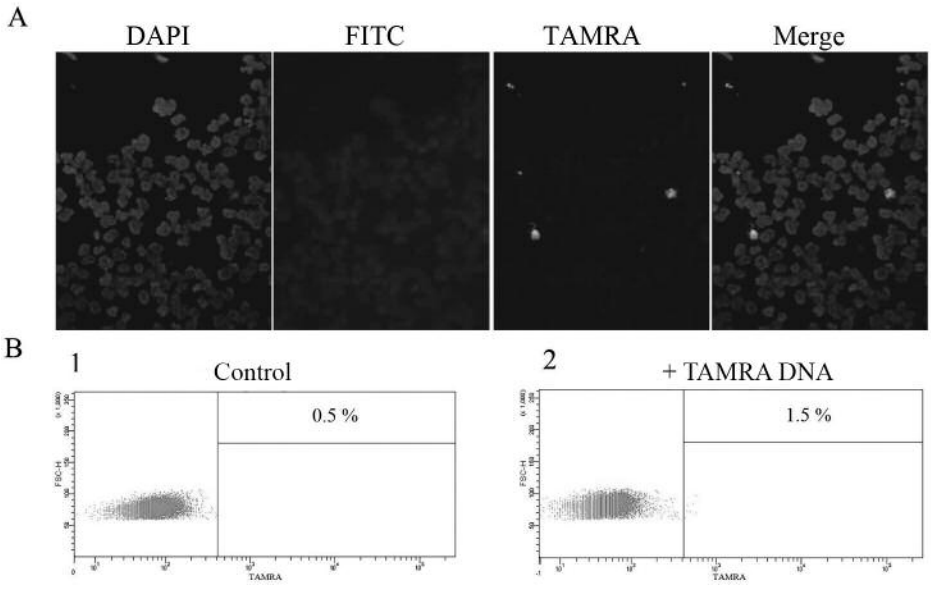

Figure 1. *Internalization of TAMRA-labeled DNA probe by RLS murine ascitic lymphosarcoma cells. A. Quantification of TAMRA-positive cells in RLS ascites using fluorescence microscopy. Arrows indicate TAMRA-positive cells. B. FACS analysis of RLS ascites cells. 1 – untreated ascitic cells (control), 2 – ascitic cells incubated with TAMRA-labeled human AluI PCR product (0.2 μg per 5×10^5 cells).*

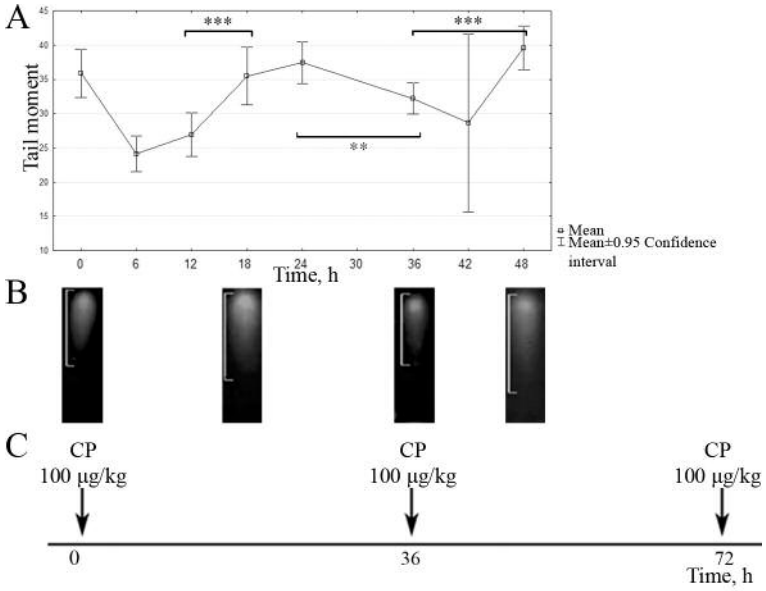

Figure 2. *Analysis of CP-induced ICLs repair process in tumor cells by the "comet tails" method. A. Time profile of the appearance and disappearance of double-strand breaks in RLS ascitic lymphosarcoma cells after single exposure to 100 μg/kg CP. The standard deviation is indicated. Results of the Student's T-test: **p<0.01; ***p<0.005. B. Images demonstrating the difference in the length of "comet tails" after electrophoresis of the nuclear material of cells in 1% agarose stained with ethidium bromide at the points of the repair process with statistically confident differences. C. The proposed schedule of CP administration based on time of ICLs repair in RLS cells.*

of the repair process in RLS ascitic lymphosarcoma cells was determined to be between 36 and 42 h (Figure 2A and B). Based on these data, the schedule for CP administration to mice was performed according to the schedule shown in Figure 2C.

RLS cells synchronization after successive therapeutic exposures to CP. The main goal of this experiment was to detect the time, when CSCs (TAMRA+ cells) in the given type of cancer exit their arrest in S/G_2/M phase caused by triple exposure to CP and

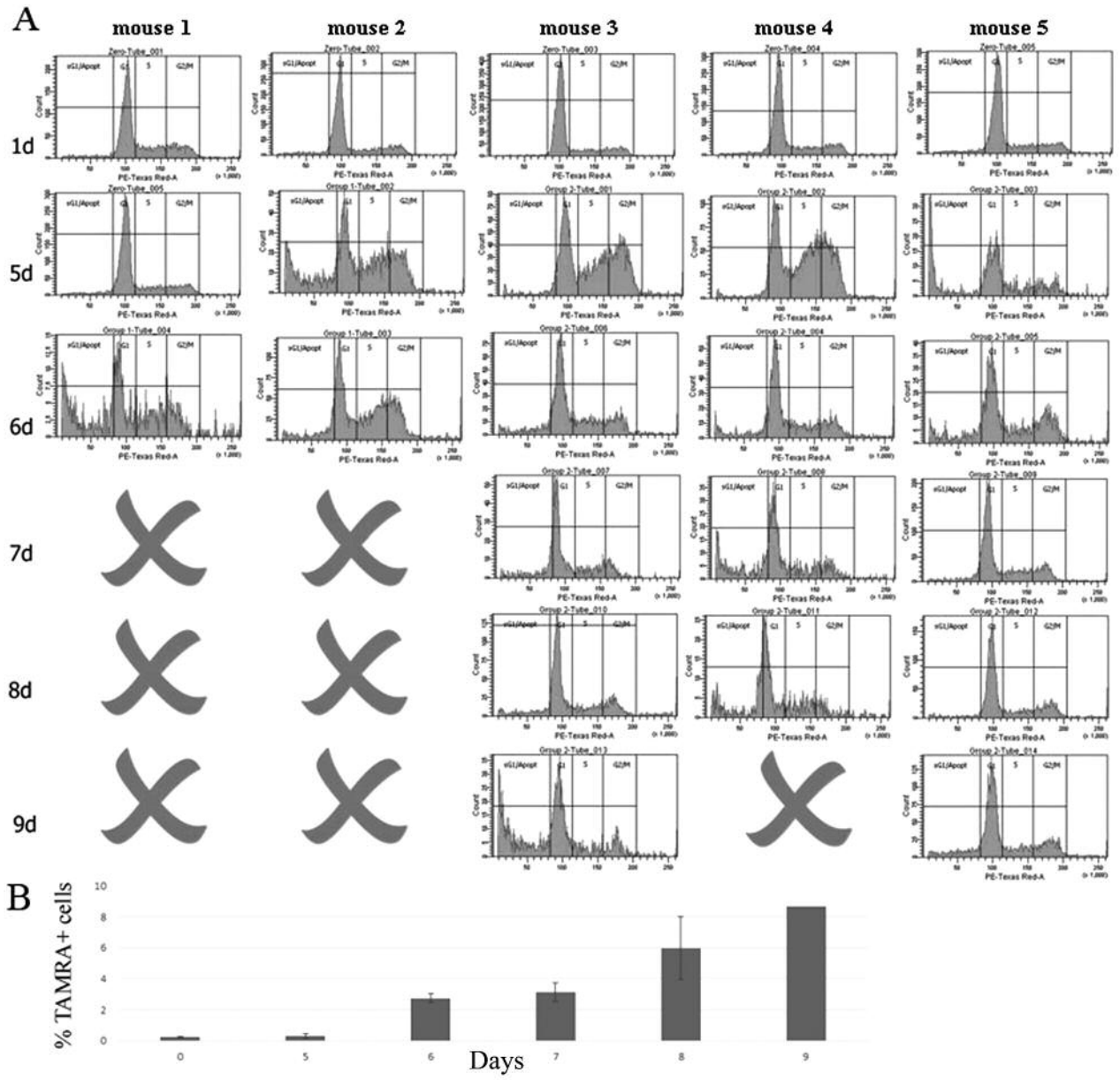

Figure 3. *Synchronization of proliferating RLS lymphosarcoma cells. A. Cell cycle profiles of RLS ascitic cells after therapeutic CP administrations with an interval of 36 h. B. Percentage of TAMRA+ cells on days 1 and 5-9 since the exposure to CP. Standard error is shown (±SE, n=3).*

synchronously enter G_1, becoming vulnerable to terminal treatment. This process is presumed to be denoted by CSCs accumulation in a certain phase of the cell cycle, which most likely would correlate with that of the bulk of tumor cells.

On days 5-6, RLS ascitic cells were found to accumulate in the late S/G_2 phase (Figure 3A). On day 7, the cell cycle profile becomes normal with the majority of cells in G_1. The percentage of TAMRA+ cells in biopsy samples (on the corresponding days) is shown in Figure 3B. On day 6, the percentage of TAMRA+ cells raised drastically (up to 3.5%) and remained stable until the next day. Further, on days 8 and 9, the percentage of TAMRA+ cells increased to 6% and 8%, respectively. At the same time, the ascites volume was notably reduced, making biopsy sampling impossible. To get statistically confident data, the experiment was repeated several times. Nevertheless, due to the high toxicity of therapeutic treatments as well as the increased aggressiveness of this type of cancer, which both caused the death of animals, it turned out to be impossible to obtain sufficient data at the "day 9" point for the correct statistical

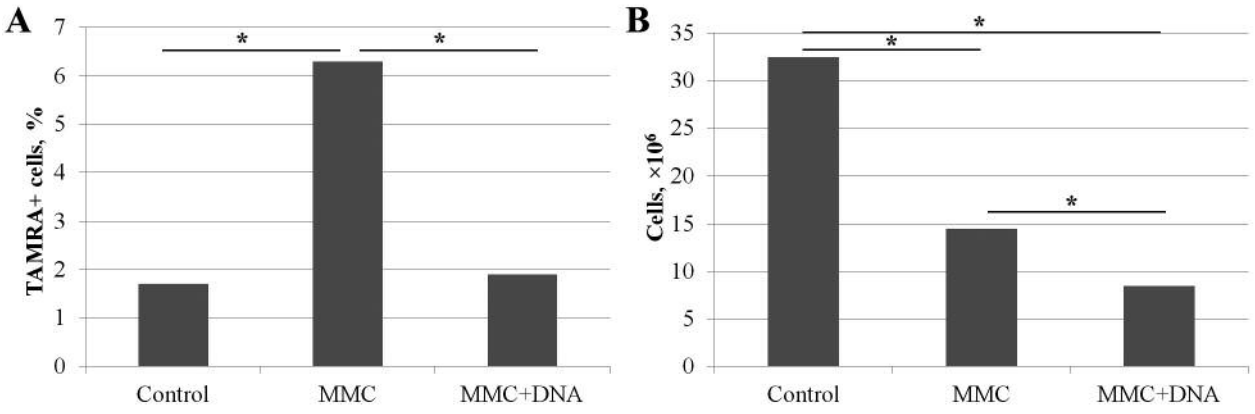

Figure 4. *Cytological analysis of the synergistic effect of a cytostatic and DNA preparation on RLS tumor cells. A. Percentage of TAMRA+ cells. B. The total number of RLS tumor cells after three times exposure to mitomycin C. *p<0.001.*

Table I. *Changes in the percentage of TAMRA+ cells after three times exposure of primary RLS tumor cell culture to mitomycin C.*

Before treatment	Cultured cells after 3 treatments				
Cell number in a well ($\times 10^6$)	Group	Total number of counted cells	Number of TAMRA+ cell	% of TAMRA+ cells	Cell number in a well ($\times 10^6$)
10	Control	11458	200	1.7	32.2
10	MMC	1812	214	6.3	14.5
10	MMC+DNA	1496	29	1.9	8.5

analysis. However, the available data analysis presumes that the general profile of cell distribution over the cell cycle since day 7 is almost identical.

The data obtained indicated day 5 to be the most suitable for the terminal treatment. Changes in the percentage of TAMRA+ cells on days 6-9 can be explained as follows. Elevation of the TAMRA+ cells percentage up to 3.5% on days 6-7 is probably associated with the beginning of large-scale apoptotic death of committed tumor cells due to their synchronous exit from cell cycle arrest with a critical number of unrepaired or incorrectly repaired DNA lesions: it is immediately before this that the massive accumulation of ascites cells in the late S/G_2 phases is observed. The stable percentage of TAMRA+ cells during these two days (days 6-7) could be due to the quiescent state of this type of cells.

The further increase to 6% and 8% on days 8 and 9 can be associated both with the ongoing apoptotic death of committed cells and with the rapid symmetrical division of survived CSCs.

Assessing the efficacy of murine RLS lymphosarcoma treatment based on the proposed "3+1" approach. Prior to "3+1" therapy, the synergistic effect of mitomycin C (MMC), which possess a crosslinking activity similar to that of CP, and dsDNA preparation on the RLS ascitic cells *ex vivo* (without the terminal treatment) was estimated. In contrast to CP, MMC directly affects cells by inducing ICLs. At the same time, the crosslinking effect of both cytostatics, *i.e.* appearance of ICLs, develops in approximately 2-4 h after exposure.

After the triple exposure of RLS cells to MMC and MMC+dsDNA, the total number of cells and the percentage of TAMRA+ cells were counted (Table I, Figure 4A and B). Triple exposure of RLS cells to MMC resulted in the death of committed ascitic cells, as is deduced from the decrease of the total number of cells per well (relatively to the control) and the simultaneous increase in the percentage of poorly differentiated TAMRA+ ones. Following MMC (as a single drug) treatment, the total number of cells per well decreased by more than two times, while the percentage of TAMRA+ cells increased by 3.7 times (Table I). MMC in combination with the dsDNA preparation induced a fourfold decrease in the total number of cells, which was due to the large-scale death of both poorly differentiated resistant CSCs and their committed progeny (decrease in the total number of cells was not associated with increase in the percentage of TAMRA+ ones). These data confirmed the overall effectiveness of the proposed approach aimed at the elimination of RLS lymphosarcoma CSCs resistant to the action of crosslinking cytostatics.

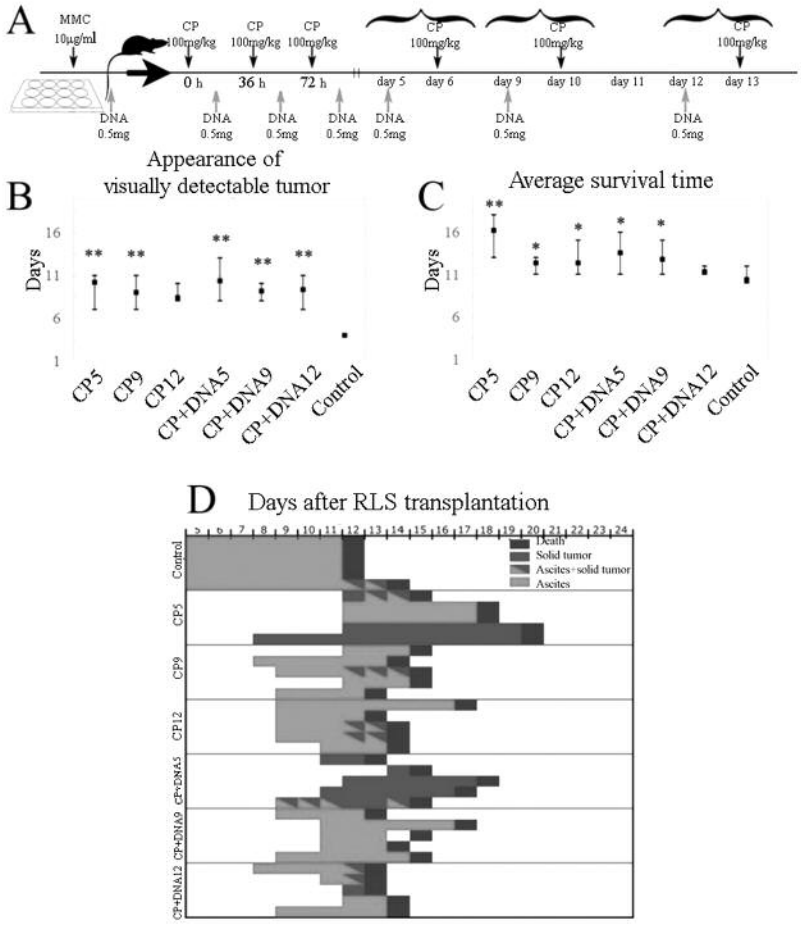

Figure 5. *Synergistic effect of CP and dsDNA preparation on the ascitic form of RLS lymphosarcoma within the proposed "3+1" approach in the in vivo experiment following in vitro pretreatment. A. The schedule of administering CP and DNA preparations within the "3+1" approach scheme (days 5, 9, 12). B. Average time of tumor growth up to visually detectable size. C. Average survival time in a group. D. The pattern of secondary tumors development in mice treated with CP and DNA preparations (both in control and experimental groups, data for each animal are given). *p<0.05; **p<0.01 relatively to the control.*

On the basis of the data on both the repair cycle length and the time of CSCs accumulation in the appropriate cell cycle phase, experiments on RLS transplantation and therapeutic treatment were carried out. To prevent carcinomatosis, mice were transplanted with RLS lymphosarcoma cells pretreated *in vitro* with mitomycin C in combination with a DNA preparation. It was presumed that such pretreatment would induce the DNA repair process, followed by cell cycle arrest, and the affected CSCs would lose their ability of invading the peritoneal tissues upon the punctures of the abdominal wall during cell transplantation. Totally, 7 groups of mice (5 animals each) were transplanted with 10^6 cells per mouse.

Therapeutic administrations of CP and dsDNA preparation were carried out in different variations of the "3+1" scheme (Figure 5A).

The results of these experiments are shown in Figure 5B-D. The applied treatments reduced the time of tumors appearance (Figure 5B). Average survival time of almost all mice treated in accordance with the given scheme, differed from that of the control group (Figure 5C). The indicative parameters (ascites reduction and average survival time) in the experimental groups, for which the terminal CP and dsDNA administration within the "3+1" scheme was performed on day 5, turned out to be better than those of other groups (Figure 5D). It should be noted that none of the therapies prevented the development of a solid tumor node in the site of the abdominal wall puncture; secondary solid tumor nodes were observed in all groups (Figure 5D).

The results of the dissection and the pathomorphological assay of the treated mice and their internal organs are shown in Figure 6. Almost all internal organs of the mice of the

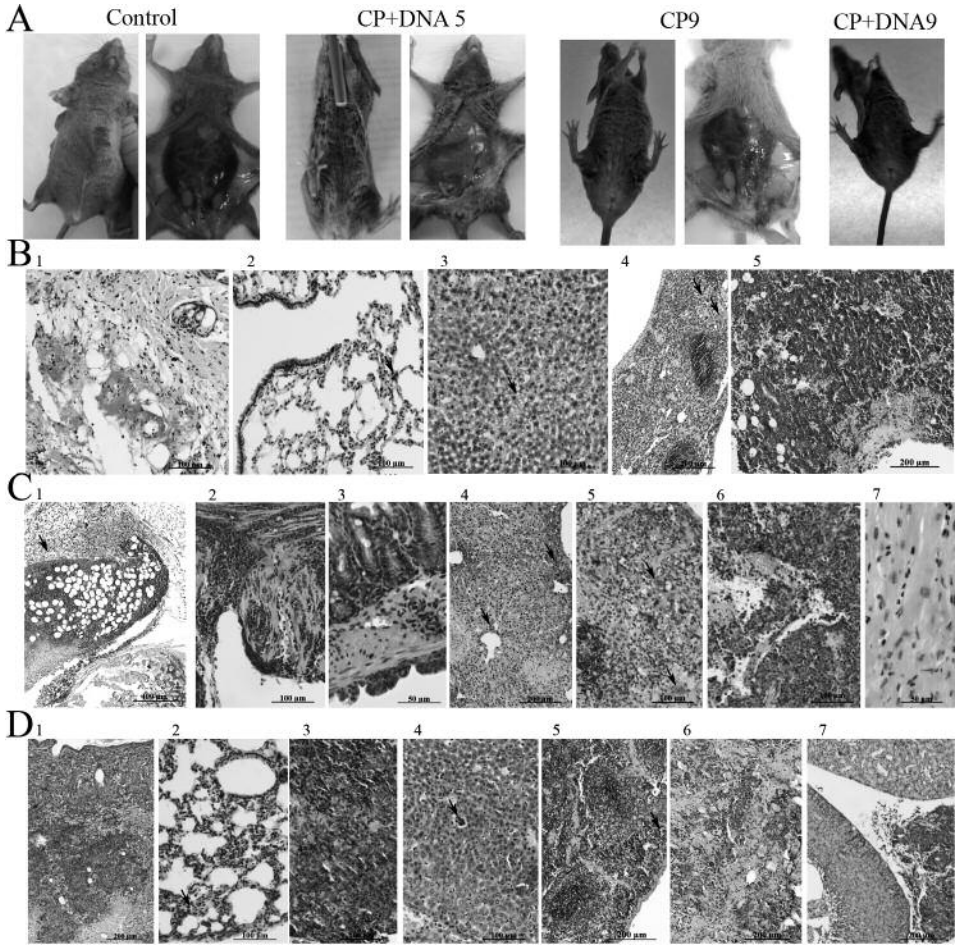

Figure 6. *Pathomorphological parameters of animals with transplanted RLS tumor after "3+1" therapy with CP and dsDNA preparation. A. Overall visual condition of experimental animals prior and after dissection. B. Pathomorphological assay of internal organs of the mouse from the CP+DNA d5 group; hematoxylin-eosin stain. 1 – Skin; the extensive necrosis site. 2 – Lungs; moderately expressed inflammation, in the projection of individual interalveolar septa there are small groups (2-3 pieces) of tumor cells. 3 – Liver; moderately expressed focal vacuole dystrophy. 4 – Spleen; small clusters of tumor cells within the organ parenchyma. 5 – Pancreas; sites with pronounced necrotic changes. C. Pathomorphological assay of internal organs of the mouse from the CP d9 group; hematoxylin-eosin stain. 1 – Skin; a completely necrotized site of the tumor implant in the reticular dermis, at the border with subcutaneous fat. 2, 3 – Small intestine; infiltration of lymphocytes and tumor cells. 4 – Liver; inflammatory infiltration, cells with morphology similar to that of tumor implant cells can be detected around the central veins and other vessels. 5 – Spleen; reduction of lymphatic follicles and small clusters of tumor cells in the red pulp. 6 – Tumor fragment in loose connective tissue. 7 – Myocardium; the overall structure corresponds to the norm, however, in certain areas, the clarity of the borders and the striated pattern of some muscle fibers is slightly blurred. D. Pathomorphological assay of internal organs of the mouse from the CP+DNA d9 group; hematoxylin-eosin stain. 1 – Skin; a large tumor node in the reticular dermis. 2 – Lungs; invasion of tumor cells into the organ parenchyma. 3 – Pancreas; substitution of glandular tissue with tumor cells. 4 – Liver; small aggregations of tumor cells in the lumen of intralobular capillaries and other vessels. 5 – Spleen; sites of different size in the red pulp with atypical implant cells infiltration, lymphatic follicles are reduced in size up to the periarterial region. 6 – Large tumor node. 7 – Kidney and adrenal gland; small foci of necrotization.*

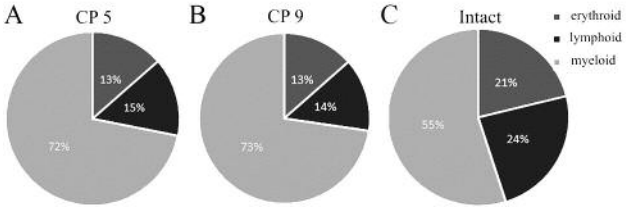

Figure 7. *Cellular composition of bone marrow. A. Mouse from the CP 5 group. B. Mouse from the CP 9 group. C. Intact mouse.*

CP+DNA 9 (Figure 6D) and CP 9 (Figure 6C) groups had foci of structural and functional lesions. At the same time, the mice of the CP+DNA 5 group had lesions in lungs, liver, spleen and pancreas only (Figure 6B). Pronounced manifestations of carcinomatosis and systemic inflammation appeared in all experimental animals.

Additionally, in CP 5 and CP 9 groups, cellular composition of the red bone marrow was assayed (Figure 7). In both experimental groups, a reduction in the percentage of erythroid and lymphoid cells accompanied by an elevation in that of myeloid ones was detected. These changes are mostly specific for systemic inflammation and testify for the development of lympho- and erythropenias.

Discussion

In the current investigation, we applied the "3+1" scheme to cure the ascitic form of murine RLS lymphosarcoma. The most effective therapeutic scheme (triple administration of CP in combination with dsDNA, which caused the arrest of tumor cells in the late S/G_2/M phases of the cell cycle and apoptosis, with terminal "strike" on day 5) provided the elimination of TAMRA+ CSCs in the RLS ascites.

RLS lymphosarcoma is known to be resistant to cyclophosphamide. Single administration of 100 mg/kg CP (the dosage generally used in the current report) caused reduction of the ascites by 16.5% only (35). Nevertheless, using the "3+1" scheme, we succeeded in curing ascites completely in the "CP+DNA/d5" group, and reducing it drastically in 40% of animals in the "CP/d5" group. It should, however, be noted that upon transplantation, RLS lymphosarcoma commonly produced solid grafts at the abdominal wall puncture site that testifies for the high metastatic ability of this tumor. Pathomorphological assay results also confirmed this conclusion. In our previous studies, we have tested the currently reported cancer treatment approach on two other ascitic tumors: Krebs-2 carcinoma and G-29 hepatocarcinoma (30, 32). In the case of Krebs-2 tumor, we have succeeded to completely cure mice from the developed ascites. The second tumor, G-29, turned out to be highly invasive. In this case, we did not succeed to cure mice completely, but we achieved the complete reduction of developed ascites. This result may have also been the result of the high invasiveness of G-29. In the case of highly invasive tumors, simple intraperitoneal injections of therapeutic agents seem to be the cause of secondary tumor foci at the abdominal wall and serous membranes puncture sites. This fact requires additional experiments on the development and testing procedures of drugs administration, which would prevent the invasion of tumor cells into the abdominal wall and serous membranes. Among the possible solutions, coating the syringe needle with a substance destroying the contacted cells may be proposed.

Ex vivo exposure of RLS cells to mitomycin C in combination with dsDNA results in proportional decrease in the number of both TAMRA+ and TAMRA- cells indicating the coincidental death of resistant CSCs and their committed progeny. Approaches in targeted treatment of CSCs are mainly limited to the detection of these cells using the surface markers specific for a certain type of tumors. Being detected, CSCs are supposed to be eliminated using specific inhibitors or other interfering agents aimed to suppression of certain signaling pathways, which determine the stem-like status of these cells, such as pluripotency and indefinite proliferative capability (40, 41). We propose the alternative approach of targeted elimination of CSCs based on the ability of this type of cells to internalize fragments of exogenous dsDNA. Being internalized, these DNA fragments disrupt the correct flow of repair of ICLs induced by exposure to crosslinking agent that, in turn, result in destruction of CSCs (42).

The currently reported death of CSCs and induction of total apoptosis of committed RLS tumor cells testify to the effectiveness of the proposed therapeutic strategy. Thus, combining all scheme elements into a single therapeutic procedure provides the possibility to eliminate a developed RLS ascites.

The determination of the time for the terminal therapeutic procedure is essential for killing the survived CSCs as has previously been shown (30-32). The present study indicated that our hypothesis was correct, and the terminal "therapeutic strike" moment is crucial for maximizing the therapeutic effect.

Being a highly aggressive lymphosarcoma, ascitic RLS produce multiple carcinomatous solid foci. Using the experimentally determined scheme of tumor cell synchronization, we succeeded to completely eliminate developed ascites in the test model. Nevertheless, solid foci, occurring, as a rule, at the sites of the abdominal wall puncture, almost always followed ascites disappearance and, along with systemic inflammation, were the cause of rapid animal death.

Introduction of the proposed approach in clinical routines, will require a precise determination of the time when tumor cells synchronously exit cell cycle arrest. As the first, step, a daily routine cell cycle profiling procedure with propidium iodide can be used to detect the accumulation of cells in the late S/G_2/M phases. Based on the results of this assay, shorter sampling times and application of more sensitive methods of cell cycle profiling, such as, for example, immunohistochemical detection of cell cycle-associated markers, should be conducted.

Conflicts of Interest

The Authors declare that they have no conflicts of interest.

Authors' Contributions

PEK carried out experiments with animals, performed the analysis and interpreted the data, SSK performed the analysis and drafted the manuscript, GSR carried out experiments with animals, YRE directed the work on microscopy, OST performed pathological analysis of animal organs and tissues and interpreted the data, TDD performed the analysis of cellular composition of bone marrow, ASP, EAP and EVD participated in the study design and carried out the molecular studies, SVS, AAO and ERC participated in the study design, SSB conceived the study, participated in its design, and coordinated the manuscript. All Authors reviewed, critically revised the final manuscript. All Authors read and approved the final manuscript.

Acknowledgements

The work was supported by the Russian Ministry of Science and High Education *via* the Institute of Cytology and Genetics (state budget project No 0324-2019-0042-C-01, state registration No AAAA-A17-117071240065-4). Microscopic analysis was supported by the State Project of the Institute of Molecular and Cellular Biology No. 0310-2019-0005.

References

1 Bonnet D and Dick JE: Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. Nat Med *3(7)*: 730-737, 1997. PMID: 9212098. DOI: 10.1038/nm0797-730

2 Efremov YR, Proskurina AS, Potter EA, Dolgova EV, Efremova OV, Taranov OS, Ostanin AA, Chernykh ER, Kolchanov NA and Bogachev SS: Cancer stem cells: emergent nature of tumor emergency. Front Genet *9*: 544, 2018. PMID: 30505319. DOI: 10.3389/fgene.2018.00544

3 Brognaro E: "The development tumor model" to study and monitor the entire progression of both primary and metastatic tumors. Tumor Biol *35(3)*: 2219-2230, 2014. PMID: 24213851. DOI: 10.1007/s13277-013-1295-9

4 Wertz IE and Dixit VM: Regulation of death receptor signaling by the ubiquitin system. Cell Death Differ *17(1)*: 14-24, 2010. PMID: 19893571. DOI: 10.1038/cdd.2009.168

5 Witsch E, Sela M and Yarden Y: Roles for growth factors in cancer progression. Physiology *25(2)*: 85-101, 2010. PMID: 20430953. DOI: 10.1152/physiol.00045.2009

6 Artandi SE and DePinho RA: Telomeres and telomerase in cancer. Carcinogenesis *31(1)*: 9-18, 2010. PMID: 19887512. DOI: 10.1093/carcin/bgp268

7 Collado M and Serrano M: Senescence in tumours: evidence from mice and humans. Nat Rev Cancer *10(1)*: 51-57, 2010. PMID: 20029423. DOI: 10.1038/nrc2772

8 Gupta RG and Somer RA: Intratumor heterogeneity: novel approaches for resolving genomic architecture and clonal evolution. Mol Cancer Res *15(9)*: 1127-1137, 2017. PMID: 28596419. DOI: 10.1158/1541-7786.MCR-17-0070

9 Kitao H, Iimori M, Kataoka Y, Wakasa T, Tokunaga E, Saeki H, Oki E and Maehara Y: DNA replication stress and cancer chemotherapy. Cancer Sci *109(2)*: 264-271, 2018. PMID: 29168596. DOI: 10.1111/cas.13455

10 Xu X, Chai S, Wang P, Zhang C, Yang Y, Yang Y and Wang K: Aldehyde dehydrogenases and cancer stem cells. Cancer Lett *369(1)*: 50-57, 2015. PMID: 26319899. DOI: 10.1016/j.canlet.2015.08.018

11 Begicevic RR and Falasca M: ABC transporters in cancer stem cells: beyond chemoresistance. Int J Mol Sci *18(11)*: 2362, 2017. PMID: 29117122. DOI: 10.3390/ijms18112362

12 Pavlides S, Whitaker-Menezes D, Castello-Cros R, Flomenberg N, Witkiewicz AK, Frank PG, Casimiro MC, Wang C, Fortina P, Addya S, Pestell RG, Martinez-Outschoorn UE, Sotgia F and Lisanti MP: The reverse Warburg effect: aerobic glycolysis in cancer associated fibroblasts and the tumor stroma. Cell Cycle *8(23)*: 3984-4001, 2009. PMID: 19923890. DOI: 10.4161/cc.8.23.10238

13 Kim SY: Cancer energy metabolism: shutting power off cancer factory. Biomol Ther (Seoul) *26(1)*: 39-44, 2018. PMID: 29212305. DOI: 10.4062/biomolther.2017.184

14 Suh DH, Kim MK, No JH, Chung HH and Song YS: Metabolic approaches to overcoming chemoresistance in ovarian cancer. Ann NY Acad Sci *1229(1)*: 53-60, 2011. PMID: 21793839. DOI: 10.1111/j.1749-6632.2011.06095.x

15 Jinesh GG and Kamat AM: Blebbishield emergency program: an apoptotic route to cellular transformation. Cell Death Differ *23(5)*: 757-758, 2016. PMID: 26967969. DOI: 10.1038/cdd.2016.26

16 Wolf K, Wu YI, Liu Y, Geiger J, Tam E, Overall C, Stack MS and Friedl P: Multi-step pericellular proteolysis controls the transition from individual to collective cancer cell invasion. Nat Cell Biol *9(8)*: 893-904, 2007. PMID: 17618273. DOI: 10.1038/ncb1616

17 Friedl P and Wolf K: Tube travel: the role of proteases in Individual and collective cancer cell invasion. Cancer Res *68(18)*: 7247-7249, 2008. PMID: 18794108. DOI: 10.1158/0008-5472.CAN-08-0784

18 Parri M and Chiarugi P: Rac and Rho GTPases in cancer cell motility control. Cell Commun Signal *8(1)*: 23, 2010. PMID: 20822528. DOI: 10.1186/1478-811X-8-23

19 Chen Q, Jin M, Yang F, Zhu J, Xiao Q and Zhang L: Matrix metalloproteinases: inflammatory regulators of cell behaviors in vascular formation and remodeling. Mediators Inflamm *2013*: 1-14, 2013. PMID: 23840100. DOI: 10.1155/2013/928315

20 Lucas B de, Pérez LM and Gálvez BG: Importance and regulation of adult stem cell migration. J Cell Mol Med *20(10)*: 1-9, 2017. PMID: 29214727. DOI: 10.1111/jcmm.13422

21 He Q, Zhang M, Zhang J, Zhong S, Liu Y, Shen J, He J, Jiang L, Yang C, Zeng Y, Guo M, Chen X, He J and Liang W: Predictive value of BRCA1 expression on the efficacy of chemotherapy based on anti-microtubule agents: a pooled analysis across different malignancies and agents. Ann Transl Med *4(6)*: 110, 2016. PMID: 27127763. DOI: 10.21037/atm.2016.03.27

22 Donahue TR and Dawson DW: Nodal/Activin signaling: a novel target for pancreatic cancer stem cell therapy. Cell Stem Cell *9(5)*: 383-384, 2011. PMID: 22056131. DOI: 10.1016/j.stem.2011.10.006

23 Kaiser J: The cancer stem cell gamble. Science 347(6219): 226-229, 2015. PMID: 25593170. DOI: 10.1126/science.347.6219.226

24 Shukla V, Rao M, Zhang H, Beers J, Wangsa D, Danny Wangsa D, Buishand FO, Wang Y, Yu Z, Stevenson H, Reardon E, McLoughlin KC, Kaufman A, Payabyab E, Hong JA, Zhang M, Sean R. Davis SR, Edelman DC, Chen G, Miettinen M, Restifo N, Ried T, Paul S. Meltzer PS and Schrump DS: Identification

of novel targets for lung cancer therapy using an induced pluripotent stem cell model. Ann Am Thorac Soc *(Suppl 2)*: S127-S128, 2018. DOI: 10.1513/AnnalsATS.201707-610MG

25 Wilson GS, Hu Z, Duan W, Tian A, Wang XM, McLeod D, Lam V, George J and Qiao L: Efficacy of using cancer stem cell markers in isolating and characterizing liver cancer stem cells. Stem Cells Dev *22(19)*: 2655-2664, 2013. PMID: 23638793. DOI: 10.1089/scd.2012.0703

26 Dolgova EV, Proskurina AS, Nikolin VP, Popova NA, Alyamkina EA, Orishchenko KE, Rogachev VA, Efremov YR, Dubatolova TD, Prokopenko AV, Chernykh ER, Ostanin AA, Taranov OS, Omigov VV, Zagrebelniy SN, Bogachev SS and Shurdov MA: "Delayed death" phenomenon: a synergistic action of cyclophosphamide and exogenous DNA. Gene *495(2)*: 134-145, 2012. PMID: 22227496. DOI: 10.1016/j.gene.2011.12.032

27 Dolgova EV, Efremov YR, Orishchenko KE, Andrushkevich OM, Alyamkina EA, Proskurina AS, Bayborodin SI, Nikolin VP, Popova NA, Chernykh ER, Ostanin AA, Taranov OS, Omigov VV, Minkevich AM, Rogachev VA, Bogachev SS and Shurdov MA: Delivery and processing of exogenous double-stranded DNA in mouse CD34+ hematopoietic progenitor cells and their cell cycle changes upon combined treatment with cyclophosphamide and double-stranded DNA. Gene *528(2)*: 74-83, 2013. DOI: 10.1016/j.gene.2013.06.058

28 Dolgova EV, Alyamkina EA, Efremov YR, Nikolin VP, Popova NA, Tyrinova TV, Kozel AV, Minkevich AM, Andrushkevich OM, Zavyalov EL, Romaschenko AV, Bayborodin SI, Taranov OS, Omigov VV, Shevela EY, Stupak VV, Mishinov SV, Rogachev VA, Proskurina AS, Mayorov VI, Shurdov MA, Ostanin AA, Chernykh ER and Bogachev SS: Identification of cancer stem cells and a strategy for their elimination. Cancer Biol Ther *15(10)*: 1378-1394, 2014. PMID: 25117082. DOI: 10.4161/cbt.29854

29 Dolgova EV, Potter EA, Proskurina AS, Minkevich AM, Chernych ER, Ostanin AA, Efremov YR, Bayborodin SI, Nikolin VP, Popova NA, Kolchanov NA and Bogachev SS: Properties of internalization factors contributing to the uptake of extracellular DNA into tumor-initiating stem cells of mouse Krebs-2 cell line. Stem Cell Res Ther *7(1)*: 76, 2016. PMID: 27225522. DOI: 0.1186/s13287-016-0338-8

30 Potter EA, Dolgova EV, Proskurina AS, Minkevich AM, Efremov YR, Taranov OS, Omigov VV, Nikolin VP, Popova NA, Bayborodin SI, Ostanin AA, Chernykh ER, Kolchanov NA, Shurdov MA and Bogachev SS: A strategy to eradicate well-developed Krebs-2 ascites in mice. Oncotarget *7(10)*: 11580-11594, 2016. PMID: 26872383. DOI: 10.18632/oncotarget.7311

31 Potter EA, Proskurina AS, Ritter GS, Dolgova EV, Nikolin VP, Popova NA, Taranov OS, Efremov YR, Bayborodin SI, Ostanin AA, Chernych ER, Kolvanov NA and Bogachev SS: Efficacy of a new cancer treatment strategy based on eradication of tumor-initiating stem cells in a mouse model of Krebs-2 solid adenocarcinoma. Oncotarget *9(47)*: 28486-28499, 2018. PMID: 29983875. DOI: 10.18632/oncotarget.25503

32 Potter EA, Ritter GS, Dolgova EV, Proskurina AS, Kisaretova PE, Efremov YR, Nikolin VP, Popova NA, Taranov OS, Ostanin AA, Chernych ER, Sidorov SV, Kolchanov NA and Bogachev SS: Evaluating the efficiency of the tumor-initiating stem cells eradication strategy on the example of ascite form of mouse gepatocarcinoma G-29. Voprosy onkologii *64(6)*: 818-829, 2018.

33 Dolgova EV, Mishinov SV, Proskurina AS, Potter EA, Efremov YR, Bayborodin SI, Tyrinova TV, Stupak VV, Ostatin AA, Chernykh ER and Bogachev SS: Novel cancer stem marker and its applicability for grading primary human gliomas. Technol Cancer Res Treat *17*: 1533034617753812, 2018. PMID: 29375020. DOI: 10.1177/1533034617753812

34 Dolgova EV, Evdokimov AN, Proskurina AS, Efremov YR, Bayborodin SI, Potter EA, Popov AA, Petruseva IO, Lavrik OI and Bogachev SS: Double-stranded DNA fragments bearing unrepairable lesions and their internalization into mouse Krebs-2 carcinoma cells. Nucleic Acid Ther *29(5)*: 278-290, 2019. DOI: 10.1089/nat.2019.0786

35 Mironova N, Shklyaeva O, Andreeva E, Popova N, Kaledin V, Nikolin V, Vlassov V and Zenkova M: Animal model of drug-resistant tumor progression. Ann NY Acad Sci *1091*: 490-500, 2006. PMID: 17341638. DOI: 10.1196/annals.1378.090

36 Patutina OA, Mironova NL, Ryabchikova EI, Popova NA, Nikolin VP, Kaledin VI, Vlassov VV and Zenkova MA: Tumoricidal activity of RNase A and DNase I. Acta Naturae *2(1)*: 88-94, 2010. PMID: 22649632.

37 Patutina OA, Mironova NL, Ryabchikova EI, Popova NA, Nikolin VP, Kaledin VI, Vlassov VV and Zenkova MA: Inhibition of metastasis development by daily administration of ultralow doses of RNase A and DNase I. Biochimie *93(4)*: 689-696, 2011. PMID: 21194552. DOI: 10.1016/j.biochi.2010.12.011

38 Grishanova AY, Melnikova EV, Kaledin VI, Nikolin VP and Lyakhovich VV: Possible role of P-glycoprotein in cyclophosphamide resistance of transplanted mouse RLS lymphosarcoma. Bull Exp Biol Med *139(5)*: 611-614, 2005. PMID: 16224562.

39 Sen'kova AV, Ageeva TA and Zenkova MA: Morphologic changes in the tumor and liver in mice with transplanted RLS40 lymphosarcoma during increase of its drug resistance. Bull Exp Biol Med *149(2)*: 258-261, 2010. PMID: 21113505.

40 Sordillo PS and Helson L: Curcumin and cancer stem cells: curcumin has asymmetrical effects on cancer and normal stem cells. Anticancer Res *35(2)*: 599-614, 2015. PMID: 25667437.

41 Moselhy J, Srinivasan S, Ankem MK and Damodaran C: Natural products that target cancer stem cells. Anticancer Res *35(11)*: 5773-5788, 2015. PMID: 26503998.

42 Romanenko MV, Dolgova EV, Osipov ID, Ritter GS, Sizova MS, Proskurina AS, Efremov YR, Bayborodin SI, Potter EA, Taranov OS, Omigov VV, Kochneva GV, Grazhdantseva AA, Zavyalov EL, Razumov IA, Netesov SV and Bogachev SS: Oncolytic effect of adenoviruses serotypes 5 and 6 against U87 glioblastoma cancer stem cells. Anticancer Res *39(11)*: 6073-6086, 2019. PMID: 31704835. DOI: 10.21873/anticanres.13815

Received December 3, 2019
Revised January 16, 2020
Accepted January 17, 2020