Karanahan: A Potential New Treatment Option for Human Breast Cancer and Its Validation in a Clinical Setting

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ABSTRACT

INTRODUCTION: *Karanahan*, a cancer treatment technology aimed at eradicating tumor-initiating stem cells, has already proven effective in 7 tumor models. *Karanahan* comprises the following procedures: (1) collecting surgical specimens, (2) determining the duration of the DNA repair process in tumor cells exposed to a cross-linking cytostatic agent, and (3) determining the time point, when cells, including tumor-initiating stem cells, are synchronized in the certain phase of the cell cycle after triple exposure to the cytostatic, becoming vulnerable for the terminal treatment, which is supposed to completely eliminate the rest of survived tumor-initiating stem cells. Determining these basic tumor properties allows to design the schedule for the administration of a cross-linking cytostatic and a complex composite DNA preparation. Being conducted in accordance with the schedule designed, *Karanahan* results in the large-scale apoptosis of tumor cells with elimination of tumor-initiating stem cells.

METHODS: Breast tumor specimens were obtained from patients, and basic tumor properties essential for conducting *Karanahan* therapy were determined.

RESULTS: We report the first use of *Karanahan* in patients diagnosed with breast cancer. Technical details of handling surgical specimens for determining the essential *Karanahan* parameters (tumor volume, cell number, cell proliferation status, etc) have been worked out. The terminally ill patient, who was undergoing palliative treatment and whose tumor specimen matched the required criteria, received a complete course of *Karanahan*.

CONCLUSIONS: The results of the treatment conducted indicate that *Karanahan* technology has a therapeutic potency and can be used as a breast cancer treatment option.

KEYWORDS: Tumor-initiating stem cells, cyclophosphamide, mitomycin C, complex composite double-stranded DNA preparation, primary breast cancer cell lines

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Introduction

Breast cancer (BC) is among the 5 most common malignant tumors, which also comprise skin tumors, lung cancer, colorectal cancer, and gastric cancer, and is the leading cause of cancer death among women worldwide, suggesting its high social importance.[1] According to the current paradigm, BC is a systemic process, with the almost synchronous growth of both the primary tumor and metastatic nodes. In more than half of the cases, BC from the very onset demonstrates aggressive development, when even tumors as small as 1 cm in size are capable of metastasizing.[2] Breast cancer constitutes a heterogeneous group of tumors differing in morphology, clinical course, and sensitivity to treatment.

The BCs are classified based on the expression of estrogen, progesterone, and human epidermal growth factor receptor 2 (HER2) receptors, Ki-67 index, tumor size, and number of lymph node metastases. In addition, there are BC subtypes distinguishable by the Prediction Analysis of Microarray 50 test.[3] According to modern concepts, the following types of BC can be clearly distinguished by their biological properties: (1) hormone-sensitive BCs, positive for estrogen and/or progesterone receptors (luminal subtypes), (2) tumors overexpressing the HER2, and (3) so-called triple-negative tumors, which express neither estrogen/progesterone receptors nor HER2 protein.[4-8]

A good prognosis for a favorable treatment outcome in BC depends on early diagnosis of the disease and the correct choice

of systemic therapy. Antitumor therapy includes radiation therapy, surgical removal of the tumor, chemotherapy, and hormone therapy, to mention the most common. Classically, the treatment scheme is as follows: first, radiation therapy; second, surgery; and finally, chemotherapy in combination with hormone drugs. When patients have HER2/neu mutations, specific targeted therapy is prescribed.[9]

The choice of BC treatment type depends directly on the stage of the disease. Despite the large number of BC treatment options developed, the most common are still surgical removal of the tumor, radiotherapy, and chemotherapy.

At present, chemotherapy in patients with BC and, in particular, with locally advanced (stage III) BC relies on the combined effect of an alkylating cytostatic agent (cyclophosphamide [CP]), anthracyclines (doxorubicin, farmorubicin, mitoxantrone), and antimetabolites (5-fluorouracil, ftorafur, gemcitabine, capecitabine, methotrexate). If necessary, treatments are supplemented with vinca alkaloids (vincristine, vinblastine, vinorelbine), taxanes (paclitaxel, docetaxel), and platinum-based drugs (cisplatin, carboplatin). The basic BC treatment schemes are FAC (combination of 5-fluorouracil, doxorubicin, and CP) or AC (doxorubicin plus CP), the efficacy of which can be enhanced by adding more components or by replacing some of the current ones with other active substances that act by different mechanisms.[10-15]

Because BC is a systemic disease, the essential treatment option is cytoreductive adjuvant or neoadjuvant chemotherapy. Adjuvant therapy for BC is supposed to eliminate the rest of tumor cells remaining after surgery in areas adjacent to the site of resection, as well as all metastases, which are thought to accompany the primary tumor growth. Neoadjuvant (primary/inductive) chemotherapy is an option for treating locally advanced and edematous-infiltrative inoperable BC cases, and its major purpose is to reduce tumor size making it sensitive to locoregional affections, such as surgery and/or radiation therapy.[16] Despite the fact that large-scale comparative randomized studies have not revealed any statistically significant differences between adjuvant and neoadjuvant chemotherapy impact on overall 5- or 9-year survival rates (European Organization for Research and Treatment of Cancer trial 10902; National Surgical Adjuvant Breast and Bowel Project B-18),[17] neoadjuvant one provided a higher relapse-free survival in patients with complete morphological regression of the tumor, especially in those under 49 years who old received this chemotherapy prior to surgery. Thus, recent research studies indicate the response to neoadjuvant chemotherapy to be largely determined by the biological characteristics of the tumor.[18,19]

The highest priority of clinical oncology practice is to enhance the efficacy of treatments, which largely depends on how sensitive to a particular treatment the tumor is. Cancer stem cells are responsible for metastasis, disease progression, and relapses after treatment. The development of methods for the enhancement of the sensitivity of exactly these cells to therapy is a key issue for oncological science.[20]

The hypothesis about the existence of a stem or tumor-initiating cell first appeared in works by Lapidot et al[21] and Bonnet and Dick.[22] This cell type has since been found in and characterized for a large number of tumor types.[23-35] Breast cancer stem cells were first isolated in 2003. A high surface expression of the epithelial specific antigen (ESA) and CD44, as well as lack or a low expression of CD24 were observed in these cells.[36] Human ESA+/CD44+/CD24– and CD44+/CD24– BC cell subpopulations have been proven to be capable of self-renewal, self-maintenance, and colony formation. Further clinical research showed that the more CD44+/CD24– BC stem cells in the primary tumor, the lower overall and recurrence-free survival rates.[37] The studies conducted revealed that the content of cancer stem cells varies in tumors with different molecular phenotypes: 1.1 ± 0.2 per 1000 cells in the luminal A subtype (estrogen receptor [ER]+ and/or progesterone receptor [PR]+, HER2/neu–); 1.3 ± 0.1 per 1000 cells in the luminal B subtype (ER+ and/or PR+, and HER2/neu+); 8.6 ± 1.0 per 1000 cells in the HER2 + subtype (ER–, PR–, HER2/neu+); 22.4 ± 1.2 per 1000 cells in the basal-like subtype (ER–, PR–, HER2/neu–, CK5/CK14+); and 17.7 ± 2.0 per 1000 cells in the normal-like subtype (ER–, PR–, HER2/neu–, CK5/CK14–). The largest number of cancer stem cells is found in tumor specimens of triple-negative molecular subtype, which have the poorest prognosis, suggesting that the number of these cells can be used not only for predicting prognosis but also for choosing the treatment option.[38-40] Overall, the above data are indicative of a correlation between cancer stem cell number in the primary tumor and disease prognosis. This correlation suggests that cancer stem cells are the key factor in the disease onset and course, and their elimination is presumed to determine the treatment outcome. Thus, finding a way to target and kill these cells in their native environment is likely to attract broad public interest. In this regard, the development of new cancer stem cells-targeting treatments (including those for BC) based on the cutting-edge 21st century technologies is seen to be extremely important.

Karanahan

The Laboratory of Induced Cellular Processes of the Institute of Cytology and Genetics of the Siberian Branch of the Russian Academy of Sciences (Novosibirsk, Russia) has spent years studying a synergic action of the cytostatic agent CP and the complex composite double-stranded DNA preparation (hereinafter referred to as "DNA-mix") and finally developed a new technology for cancer treatment, which was called "*Karanahan*" (from the Sanskrit *kāraṇa* ["source"] + *han* ["to kill"]), which eradicates tumor-initiating stem cells (TISCs).[41,42]

The following discoveries contributed to the development of *Karanahan* technology:

- Discovering a universal molecular marker for poorly differentiated cells (including tumor ones), namely, their

capability of native internalizing extracellular double-stranded DNA fragments that allows detecting TISCs using a tetramethylrhodamine (TAMRA)-labeled DNA probe;
- The ability of internalized DNA fragments to interfere in DNA interstrand cross-link (ICL) repair in such a manner that TISCs either die out or lose their tumorigenicity. Deprived of the source of renewal and unlimited growth, the tumor eventually succumbs to the host defenses.
- Finding the phenomenon of TISCs synchronization in the "treatment-sensitive" phase of the cell cycle, and using the determined synchronization timing for their eradication. To have TISCs synchronized, we induce 3 instances of cell cycle arrest, each by exposure to the cross-linking cytostatic agent on completion of each of the 3 consecutive DNA repair cycles. Several days after the exposure, cells accumulate in the G2/M phase of the cell cycle and proceed to G1 all at once. At this point, the final, TISC-eradicating exposure to the cross-linking cytostatic agent and DNA-mix is administered.

In summary, the timeline of exposure to a cross-linking cytostatic agent and DNA-mix under *Karanahan* is set as follows. Exposure to the cross-linking cytostatic agent is administered every time after completion of each of the 3 consecutive DNA repair cycles, at the point when there are the fewest double-strand breaks (DSBs) in the tumor cells. The fourth exposure to the cross-linking cytostatic agent is administered when cells are synchronized in G2/M due to the previous 3 exposures to the cross-linking cytostatic agent. DNA-mix is administered after each exposure to the cross-linking cytostatic agent at the point when DSBs in the cells are at a maximum and nucleotide excision repair (NER) switches to homology recombination. The principle schema for *Karanahan* implementation is shown in Supplemental Figure 1.

We have previously demonstrated[42] that the correctly timed final (or terminal) exposure eradicates TISCs and cures experimental mice even with a cross-linking cytostatic agent alone (in our case, that was CP). This treatment option can be regarded as low/medium-dose intermittent metronomic CP chemotherapy relying on the timing of repair of CP-induced DNA ICLs and the time at which TISCs become synchronized.

Antitumor effect of intermittent metronomic CP chemotherapy

Attention has recently become closely focused on the ability of CP to modulate tumor-infiltrating immune cells toward antitumor status. Cyclophosphamide administered solely at therapeutic doses causes a prolonged reduction in immune cells content in the peripheral blood, bone marrow, and spleen—for as long as 6 to 20 days.[43,44] In addition, the use of CP as part of therapy aimed at destruction of tumor cells enhances pro-tumor capabilities of the tumor-associated stroma, stimulating tumor growth, and progression.[45] Because CP targets and destroys mainly the bulk of committed tumor cells, the tumor-protecting properties of the tumor-associated stroma, which both suppresses the immune cells activity and enhances the immunologic resistance of the tumor, facilitate the survival and proliferation of chemoresistant clones originating from TISCs, which intrinsically are almost completely resistant to CP.[28,40,41,43-50] At the same time, low/medium-dose metronomic administration of CP as a monodrug exerts a stimulating effect on immune cells, especially tumor-infiltrating ones such as natural killer cells, dendritic cells, natural killer T cells, CD4+, CD8+, and B cells. It is also typical of this therapeutic approach that it deprives CD25+/FOXP3+ regulatory T cells from the tumor node and switches tumor-associated macrophage polarization from M2 to M1. The reactivation of immunocompetence leads to tumor regression.[51-54]

In summary, it can be concluded that low/medium-dose intermittent metronomic CP chemotherapy, which relies on both the timing of repairing CP-induced DNA ICLs and that of TISCs synchronization (a variant of *Karanahan*, but without the use of DNA-mix), has 3 special features to mention. Triple consecutive exposures to CP results in (1) large-scale apoptosis of the bulk of tumor cells independently on tumor type and origin[55,56]; (2) synchronization of tumor cells (including TISCs) and their accumulation in G2/M with following transition into G1, which is observed as shifts in cell cycle peaks[57] (on the day when TISCs synchronously exit the cell cycle arrest, the terminal exposure to CP, leading to either complete or significant eradication of TISCs,[58,59] is administered); and (3) depletion of immunosuppressive regulatory T cells, repolarization of myeloid-derived suppressor cells to tumor reactive one and consequent activation of effector lymphocytes.[53,54]

This study represents an effort to validate *Karanahan* as a treatment option for different BC subtypes on the clinical floor.

Methods

Karanahan schedule

Before proceeding to *Karanahan*, the following 3 issues are to be addressed: (1) the presence and content of TAMRA+ TISCs in the specimen; (2) the duration of DNA repair process after exposure to a cross-linking cytostatic agent; (3) the time point (the day) of tumor cells synchronization.

These "setup inputs" are different for each particular tumor and all have immediate relevance to decisions on *Karanahan* schedule.

Patients

Research was conducted according to the World Medical Association Declaration of Helsinki. Research was approved by the Local Ethics Committee at the Research Institute of

Fundamental and Clinical Immunology. Participants provided written informed consent. Breast tumor specimens were obtained from patients in the Novosibirsk Municipal Clinic No. 1. Patient data are provided in Table 1.

Complex composite double-stranded DNA-based preparation

Complex composite double-stranded DNA-based preparation (DNA-mix) is the combination of 3 pharmacopoeial substances included in the register of pharmacologic substances of the Russian Federation, which are to be mixed strictly as follows: (1) the preparation of fragmented salmon sperm (Derinat) (Registration certificate N 002916/01 from 27.02.2008); (2) chlormethine, a cross-linking cytostatic drug of direct action (CAS: 51-75-2); and (3) the preparation of fragmented human DNA (Panagen) (Registration certificate N LSR-004429/08 from 09.06.2008).[61] DNA-mix is the subject of industrial property of KARANAHAN LLC.

Primary cultures

Immediately after surgery, the tumor specimen was placed in Dulbecco's Modified Eagle Medium (DMEM)/F12 (1:1) supplemented with gentamicin (100 μg/mL) and amphotericin B (1 μg/mL) for transportation purposes. The tissue was sheared using a scalpel in a glass Petri dish and placed in 20 to 40 mL of DMEM/F12 (1:1) supplemented with type I collagenase (0.2%) from *Clostridium histolyticum* (Gibco, USA). The resulting sample was incubated for 2 to 3 hours at 37°C, with intermittent stirring. The suspension was strained through a 40-μm filter, cells were sedimented at 400 g for 5 minutes at 4°C, transferred to culture flasks with DMEM/F12 (1:1) medium supplemented with 10% fetal bovine serum (HyClone, USA) and gentamicin (100 μg/mL), and cultivated in a CO_2-incubator at 5% CO_2, 37°C, and 95% humidity.

TAMRA+ cell counts

After overnight cultivation under standard conditions, 0.2 million cells were incubated for an hour in a serum-free medium with TAMRA-labeled DNA (0.2 μg) at room temperature in the dark; preparations were made and examined using an Axio Imager (Zeiss) fluorescent microscope in the Core Facility Center for Microscopic Analysis of Biological Samples, SB RAS.

Distribution of tumor cells along the cell cycle

After overnight cultivation under standard conditions, 0.5 million cells were fixed in 50% methanol and stored at +4°C until used. Cells were sedimented at 400 g for 10 minutes at 4°C and incubated with RNase (200 μg/mL) and propidium iodide for 0.5 to 1 hour at 37°C. Analysis was performed using a BD FACSAria III cell sorter in the Core Facility Center for Flow Cytometry, Institute of Cytology and Genetics, SB RAS.

Duration of the DNA repair process

The duration of the DNA repair process was assessed with the use of "comet assay" after exposure of cells to 1 μg/mL of mitomycin C (Sigma, USA) for 1 hour as described in Kisaretova et al,[41] where it was experimentally confirmed that double-stranded breaks induced in cell culture by exposure to mitomycin C, and in vivo by treatment with CP, both appear in the same time frames, namely 2 hours after exposition, that allows to extrapolate the data obtained ex vivo to those expected in vivo.[41]

Microscopic analysis was performed using a Zeiss Axio Imager fluorescent microscope in the Core Facility Center for Microscopic Analysis of Biological Samples, SB RAS.

The time of cell synchronization after 3 exposures to mitomycin C

The cell culture was exposed to the cytostatic agent mitomycin C for an hour at 1 μg/mL, 3 times at chosen intervals. Cells were sampled for assessing the cell cycle distribution immediately before the first exposure (intact control sample) and on days 4 to 11 after it. The sampled cells were fixed in 50% methanol and stored at 4°C. Cell cycle distribution was assessed using propidium iodide at all experimental time points simultaneously.

Patient No. 6: a case for Karanahan

Patient No. 6 had BC recurrence. One year before this study, the patient had undergone primary treatment: radical surgery with axillary lymph node dissection, radiation therapy, and tamoxifen treatment (20 mg/day). By the beginning of this study, the patient had developed lumps in the left breast, as well as metastases in the left temporal region, inguinal lymph node, and in the liver. The tumor was dissected from the left breast; histological assay indicated invasive ductal carcinoma. A specimen of this tumor was used for addressing the "setup inputs." The patient gave written voluntary and informed consent for participation in *Karanahan* treatment. All the *Karanahan* procedures were performed by the attending physician in accordance with our recommendations. During the first course, the patient received 4 intravenous injections of CP solely at the dose of 300 mg/m^2 in full accordance with determined schedule. Twenty-one days later, the patient received the second course of 4 intravenous CP injections at the dose of 300 mg/m^2 in combination with 4 injections of 0.5 mg of DNA-mix dispersedly administered into the inguinal lymph node metastasis.

Table 1. Patient information and the main clinical features of the tumor specimens.

PATIENT NO.	AGE	STAGE	HISTOLOGY				IMMUNOHISTOCHEMISTRY				TUMOR SUBTYPE[60]
			FORM	DIFFERENTIATION LEVEL	LVI	LYMPH NODES AFFECTED	ER	PR	HER2/ NEU	KI-67, %	
1	47	dT2N1M0	IDC*	G II	+	2 of 10	3	2	3+	25	Luminal B (HER2/neu+)
2	53	dT2N1M0	IDC	G II	+	2 of 10	3	2	3+	25	Luminal B (HER2/neu+)
3	69	dT2N0M0	IDC	G II	–	–	5	6	0	18	Luminal B (HER2/neu–)
4	72	T1NxM0	IDC	G II	–	–	6	0	3+	15	Luminal B (HER2/neu+)
5	61	sT2N0M0	IDC	G II	–	–	8	5	3+	35	Luminal B (HER2/neu+)
6	57	sT2N0M1	IDC	G II	–	–	7	7	0	45	Luminal B (HER2/neu–)
7	84	sT1N0M0	IDC	G II	–	–	8	8	0	5	Luminal A
8	62	sT1N0M0	Mucinous carcinoma	G II	–	–	8	7	0	10	Luminal A
9	61	dT4NM0	IDC	G II	+	9 of 17	8	8	0	25	Luminal B (HER2/neu–)
10	71	dT1N0M0	IDC	G II	–	–	3	3	0	95	Luminal B (HER2/neu–)
11	67	sT2N1M0	IDC	G II	–	1 of 8	8	7	3+	25	Luminal B (HER2/neu+)
12	60	sT4N1M0					6	6	0	10	Luminal A
13	61	sT1NxM0	IDC	G II	+	6 of 8	7	5	0	13	Luminal A
14	62	sT1NxM0 dT1NxM0	IDC	G II	–	1 of 10	8	7	0	70	Luminal B (HER2/neu–)
15	63	dT2N1M0	IDC	G II	+	2 of 12	7	5	0	20	Luminal B (HER2/neu–)
16	51	dT2N1M0	IDC	G II	–	3 of 14	7	3	0	10	Luminal A
17	66	sT2NxM0	IDC	G II	–	–	0	0	0	30	Triple-negative
18	68	dT2NxM0	IDC	G II	–	–	8	0	3+	15	Luminal B (HER2/neu+)
19	53	dT2NxM0	IDC	G II	–	–	3	2	0	85	Luminal B (HER2/neu–)
20	72	sT1NxM0	IDC	G II	+	3 of 10	8	8	1+	20	Luminal B (HER2/neu+)
21	61	dT2N1M1	IDC	G II	+		0	0	3+	23	Nonluminal HER2/neu+
22	64	sT2NxM0					2	0	3+	30	Luminal B (HER2/neu+)
23	63	dT2NxM1					8	8	0	20	Luminal B (HER2/neu–)
24	59	sT1NxM0	IDC	G II	+	3 of 10	5	1	0	10	Luminal A

(Continued)

Table 1. (Continued)

PATIENT NO.	AGE	STAGE	HISTOLOGY				IMMUNOHISTOCHEMISTRY				TUMOR SUBTYPE[60]
			FORM	DIFFERENTIATION LEVEL	LVI	LYMPH NODES AFFECTED	ER	PR	HER2/ NEU	KI-67, %	
25	50	sT4N3M0	IDC	G II	–	–	7	3	0	75	Luminal B (HER2/neu–)
26	57	sT4N3M0					8	8	0	10	Luminal A
27	45	dT2NxM0					8	7	0	35	Luminal B (HER2/neu–)
28	56	sT2NxM0					0	0	0	85	Triple-negative
29	72	dT1NxM0					8	8	0	10	Luminal A
30	58	dT2NxM0					8	7	0	15	Luminal B (HER2/neu–)
31	48	sT2NxM1 dT3N2M1					8 8	8 4	3+ 3+	15 20	Luminal B (HER2/neu+)
32	53	dT1NxM0	IDC	G II	–	1 of 10	8	5	1+	20	Luminal B (HER2/neu+)
33	64	dT2NxM0	IDC	In situ	–	–	8	8	3+	35	Luminal B (HER2/neu+)
34	48	sT2NxM0	IDC	G II	+	–	0	0	0	5	Triple-negative

Abbreviations: ER, estrogen receptor; HER2, human epidermal growth factor receptor 2; IDC, invasive ductal carcinoma; LVI, lymphovascular invasion; PR, progesterone receptor.

Results

Before implementation of *Karanahan* into a clinical practice, it was required to ensure the surgical specimens are suitable for determining all the essential *Karanahan* parameters (see section "Methods"). Being determined, these parameters are used to design the schedule of CP and DNA-mix administration.

Initially, this study was expected to result in a large-scale clinical use of the novel technological approach. However, we have encountered multiple biological and progression-related tumor-specific factors that it was decided to confine the study to a single patient with disseminated BC.

Cells from 34 surgery specimens of BC were assayed and the results were systematized. This allowed us to estimate the factors affecting the applicability of *Karanahan* to different types of BC and to reveal some peculiarities of this neoplasia.

BC specimen summary

Table 1 contains basic information about the patients and the main clinical features of the tumor specimens. As can be seen from the table, the tumor specimens were from patients with stage II to IV disease.

Characterization of some biological features of BC cells isolated from surgery specimens

The presence of TAMRA+ TISCs in primary cultures derived from BC specimens. Six primary cell cultures were analyzed. All of them contained TAMRA+ cells (Figure 1A). Microscope assays revealed that TAMRA+ cells made up from 0.3% to 5.5% of BC cells in primary cultures (Table 2).

The respective content of CD44+/CD24– cancer stem cells and TAMRA+ cells internalizing the labeled DNA probe were compared (Figure 1B and C; Table 3). It was found that TAMRA+ cells make up from ~10 to ~30% of the CD44+/CD24– cancer stem cells.

Proliferative activity of cells in BC specimens. For the accurate assessment of the proliferative activity of cancer cells, it was required to understand whether exposure to collagenase, as well as cultivation in the CO_2 incubator affect this parameter. Proliferative activity was measured in 5 specimens using propidium iodide-based Flow cytometry assay in preliminary experiments at 3 points: (1) immediately after shearing each tumor specimen with a scalpel, (2) after exposure to collagenase for 4 hours at 37°C, and (3) after cultivation in the CO_2 incubator for 24 hours under standard conditions (Figure 2A). It was demonstrated that neither exposure to collagenase nor 24-hour cultivation exerted any effect on the cell cycle profile. At the same time, the proportion of apoptotic cells in samples substantially increased in a time-dependent manner. This could be due to the deprivation of the stromal influence, which is presumed to play a pivotal role in supporting the viability of tumor cells, after collagenase treatment and in vitro cultivation.

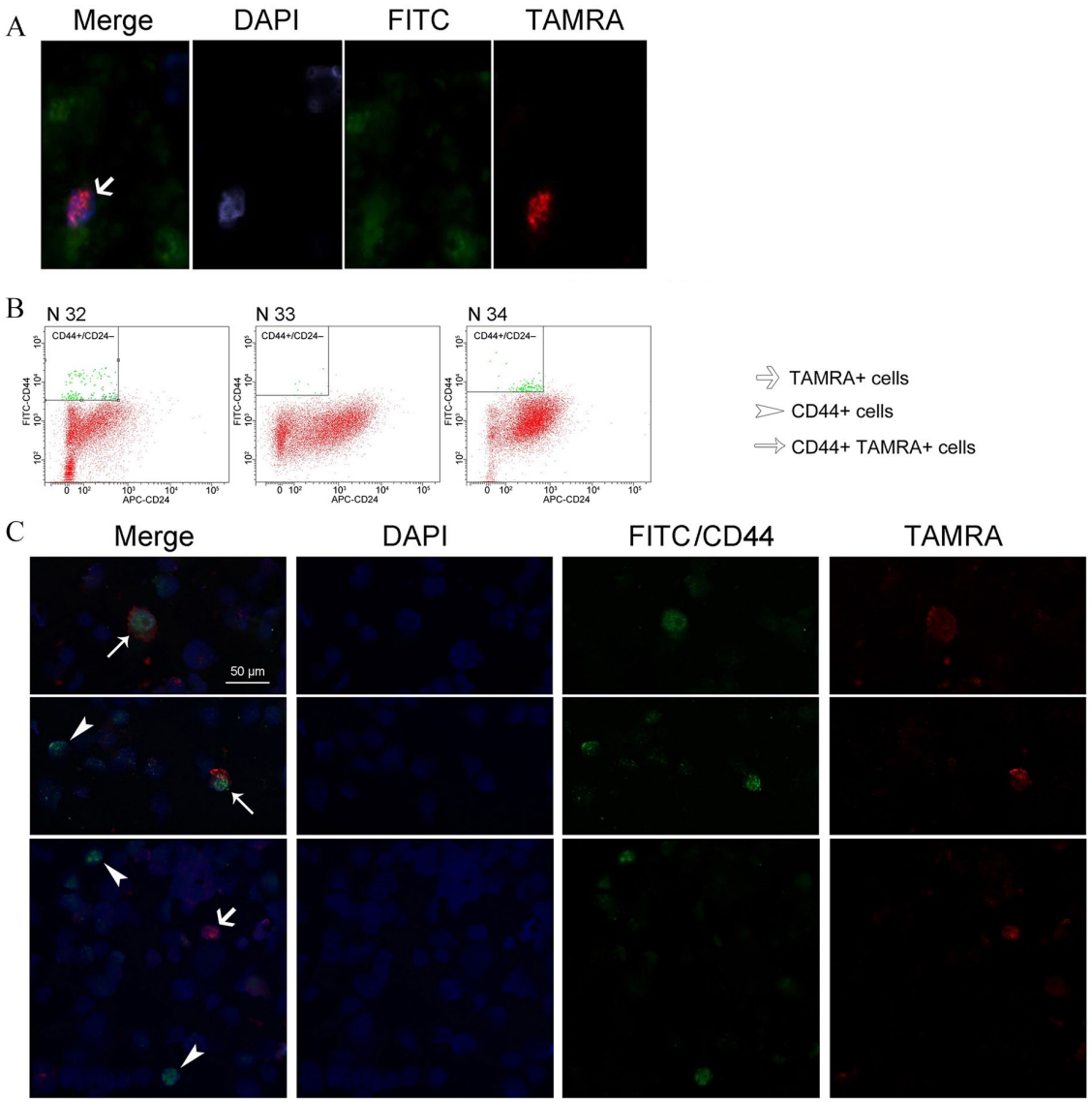

Figure 1. Detection of TISCs in primary cultures derived from BC specimens. (A) TAMRA+ TISCs. (B) Flow cytometry of 3 tumor specimens; CD44+/CD24– cancer stem cells. (C) Fluorescence microscopy of cell samples after incubation with TAMRA-labeled DNA fragments and staining with FITC-labeled CD44 antibodies. BC indicates breast cancer, FITC, fluorescein isothiocyanate; TISCs, tumor-initiating stem cells.

Table 2. Percentage of TAMRA+ cells in primary cultures of BC cells.

PATIENT NO.	TAMRA+ CELLS, %
1	5.45
2	2.19
3	1.53
4	0.31
6	0.57
31	0.50
32	0.81
33	0.35
34	0.74

Abbreviation: BC, breast cancer.

Table 3. TAMRA+ cells versus CD44+/CD24– cells, and CD44+/CD24– cells versus BC cells in primary cultures (%).

PATIENT NO.	CD44+/CD24– CELLS, %	OF THEM, TAMRA+ CELLS, %
32	1.2	33.9
33	0.1	33.3
34	0.7	8.8

Abbreviation: BC, breast cancer.

Proliferative activity was measured in 28 primary cultures of BC specimens. It was found that each tumor specimen exhibited different proliferative activity. About 60% of the primary cultures were at the state of dormancy (G1/G0), while the others, including 3 metastasis samples (nos.

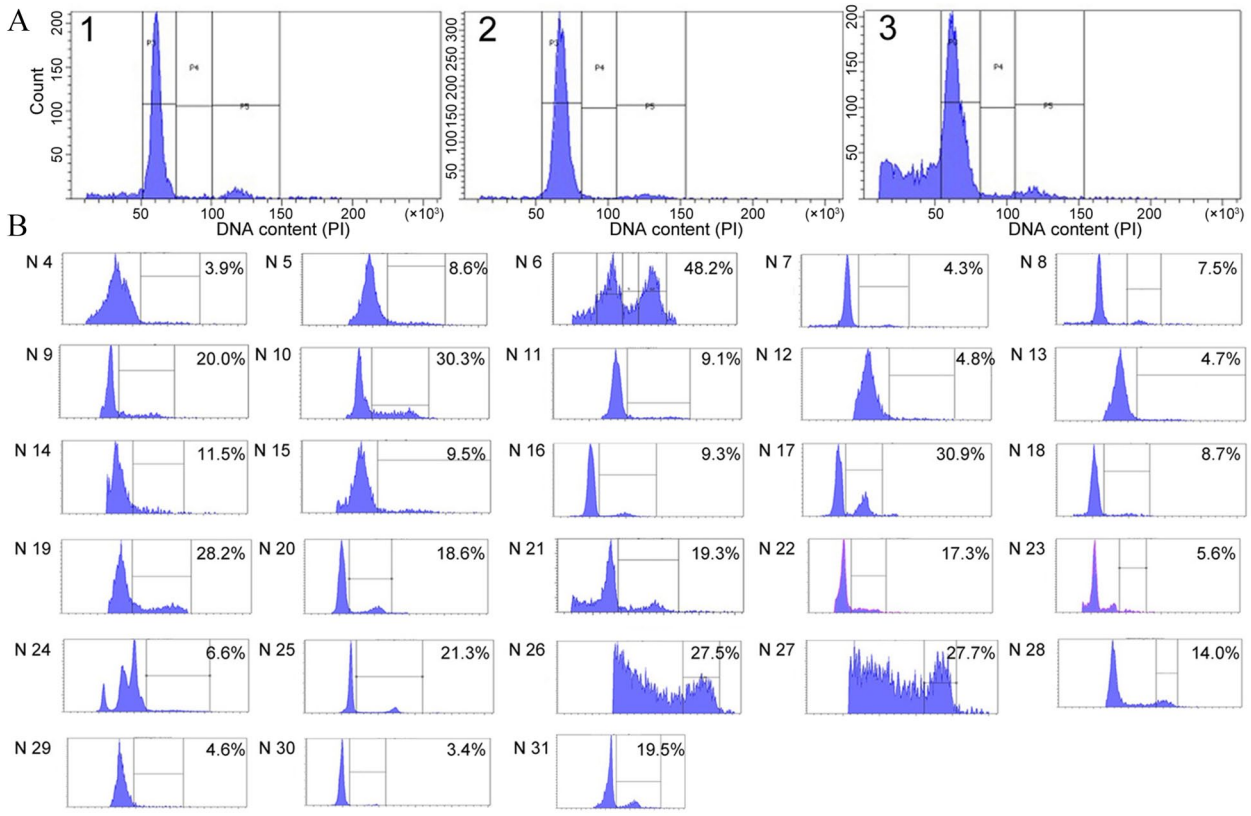

Figure 2. Assessment of the proliferative activity of BC cells by propidium iodide-based Flow cytometry. (A) Exposures and their implications for proliferative activity: (1) cells immediately after mincing, (2) cells after exposure to collagenase by incubation for 4 hours at 37°C, and (3) cells after culture in a CO_2 incubator under standard conditions. (B) Proliferative activity of BC cells in primary cultures. The X-axis represents the relative DNA content determined by the propidium iodide fluorescence, and the Y-axis represents the number of cells with the appropriate DNA content. Patient numeric IDs as well as the percentage of cells undergoing division are indicated. No additional numerical data (such as fluorescence intensity or exact number of cells) are shown because of their relative nature. BC indicates breast cancer.

6, 21, and 31), were dividing at different rates (Figure 2B). The percentage of dividing cells measured in luminal A and luminal B HER2/neu– subtype samples ranged from 3 to 50. In luminal B HER2/neu+, dividing cells make up 4% to 20%. In nonluminal HER2/neu+ samples and triple-negative BC samples, dividing cell make up 14% to 31% (Table 4).

Assessing the duration of repairing mitomycin C-induced DNA ICLs in tumor cells. In proliferating cells exposed to mitomycin C, DSBs were found to appear and disappear in a more or less standard manner, but the exact time profiles of this process differed for different specimens (Figure 3). In the case of Patient no. 5, despite the low content of proliferating cells, exposure of the culture to mitomycin C caused the standard pattern of DSBs appearance and disappearance, indicating the ongoing DNA repair process.

Estimating the proliferative status of tumor cells after a single exposure to 1 μg/mL of mitomycin C. As indicated above, the tumor specimen(s) with a low content of dividing cells (patient no. 5) displayed the pattern of DSB appearance/disappearance similar to those in actively proliferating cell cultures. To explain this phenomenon, we assumed that exposure to the cytostatic agent triggers the proliferative process.

To clarify this hypothesis, cell cycle profiles in cells after a single exposure to 1 μg/mL of mitomycin C were daily monitored for 7 days since the exposure. Proliferation was found to be activated (1.05- to 1.82-fold increase in the content of dividing cell) in 43% of the primary cultures, while the rest of them (57%) displayed the reduced proliferative activity (Figure 4).

Estimating the day of cell synchronization after 3 exposures to mitomycin C scheduled regarding the DNA repair duration. Several attempts were made to estimate the day of cell synchronization in the primary cultures of BC cells. However, we succeeded only with cells from the metastasis specimens. The other attempts failed due to a desperately low number of viable cells after their incubation for 14 days required for determining the duration of the DNA repair process and subsequently estimating the day of the terminal treatment.

Thus, it turned out that the essential *Karanahan* parameters, namely the presence of TAMRA-positive TISCs, the duration of the DNA repair process, and the time point of the terminal treatment, cannot be fully determined for all possible cases, but only for those meeting certain criteria.

Table 4. Comparison of some biological features of cells in the BC samples.

PATIENT NO.	KI-67, %	DIVIDING CELLS, S/G2/M, %	TAMRA+, %	CANCER STEM CELLS AS % OF TOTAL TUMOR CELLS[38-40]
Luminal A				
7	5	4.3	-	0.11 ± 0.02
8	10	7.5	-	
12	10	4.8	-	
13	13	4.7	-	
16	10	9.3	-	
24	10	6.6	-	
26	10	27.5	-	
29	10	4.6	-	
Luminal B HER2/neu–				
3	18	-	1.53	
6	45	48.2	0.57	
9	25	20.0	-	
10	95	30.3	-	
14	70	11.5	-	
15	20	9.5	-	
19	85	28.2	-	
23	20	3.2	-	
25	75	21.3	-	
27	35	27.7	-	
30	15	3.4	-	
Luminal B HER2/neu+				
1	25	-	5.45	0.13 ± 0.01
2	25	-	2.19	
4	15	3.9	0.31	
5	33	8.6	-	
11	25	9.1	-	
18	15	8.7	-	
20	20	18.6	-	
22	30	17.3	-	
31	20	19.5	0.5	
32	20	-	-	
33	35	-	-	
Nonluminal HER2/neu+				
21	23	19.3	-	0.86 ± 0.1
Triple-negative				
17	30	30.9	-	1.57-2.36
28	85	14.0	-	
34	5	-	-	

Abbreviations: BC, breast cancer; HER2, human epidermal growth factor receptor 2.

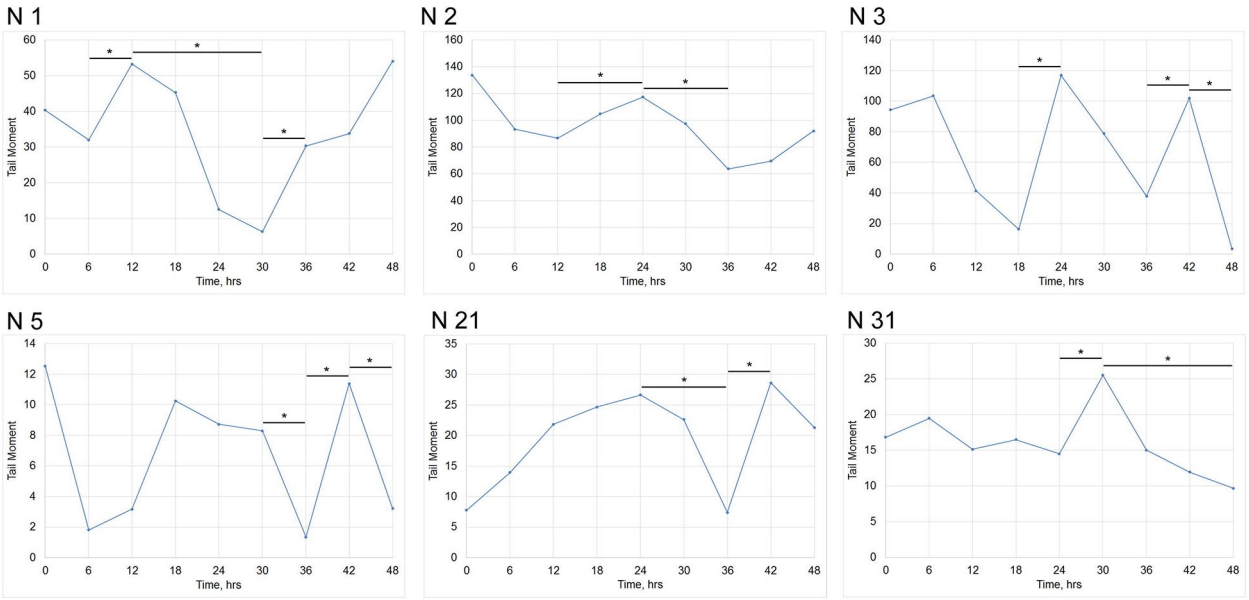

Figure 3. Repair processes in BC cells after exposure to 1 μg/mL of mitomycin C. The values are medium of tail moments in cells that is equivalent to amount of DSBs. BC indicates breast cancer; DSBs, double-strand breaks.
*Differences between points are significant at $P < .05$, Wilcoxon Mann-Whitney test.

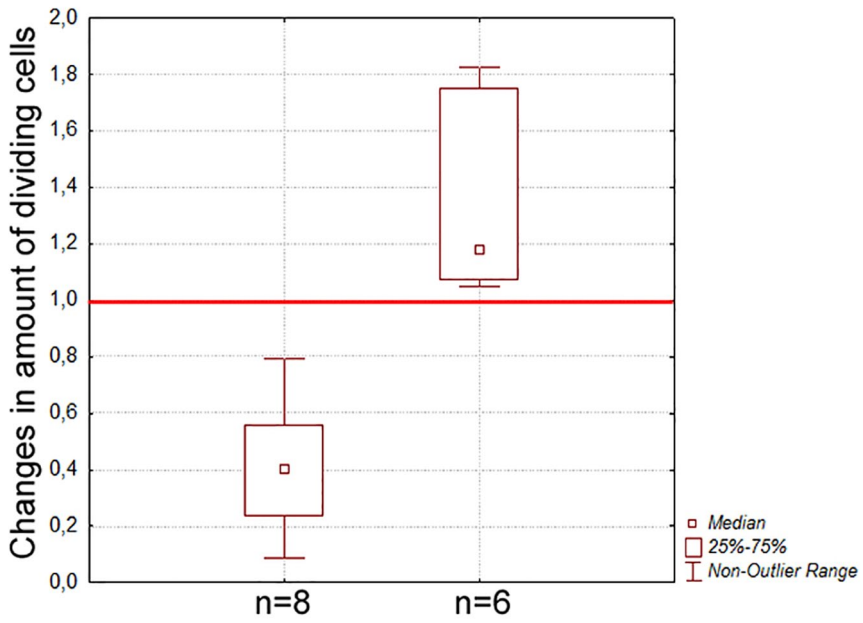

Figure 4. Changes in the content of dividing cells in primary cultures after single 1-hour exposure to 1 μg/mL of mitomycin C. In 8 patients, the content of dividing cells was decreased relative to the baseline (red line), while in other 6 ones, it was increased.

Our experience with surgery specimens led us to the conclusion that correct *Karanahan* implementation requires (1) a large amount of tumor material with (2) a high content of actively dividing tumor cells. Only abidance by these principles ensures the correct interpretation of the data obtained and implementation of *Karanahan* as a therapeutic approach.

The case of Karanahan clinical implementation

A clinical test of the technology was performed on the terminally ill palliative care patient with inoperable BC, for whom all the *Karanahan* parameters were successfully determined.

The tumor specimen was from patient no. 6 with recurrent BC. The patient also showed tumor progression with large metastases in the breast, left temporal region, left inguinal lymph node, and liver, confirmed by ultrasonic imaging and histological assay of biopsy specimens from the tumor nodes.

The percentage of cells internalizing the TAMRA-labeled DNA probe was 0.57 (Figure 5A). When determining proliferative activity, it was found that cells were dividing actively, the fraction of dividing cells constituted up to 48.2%. Based on the DNA repair timing data, exposure time points for the cell culture were chosen and set at hours 0, 28, and 56 (Figure 5B and C).

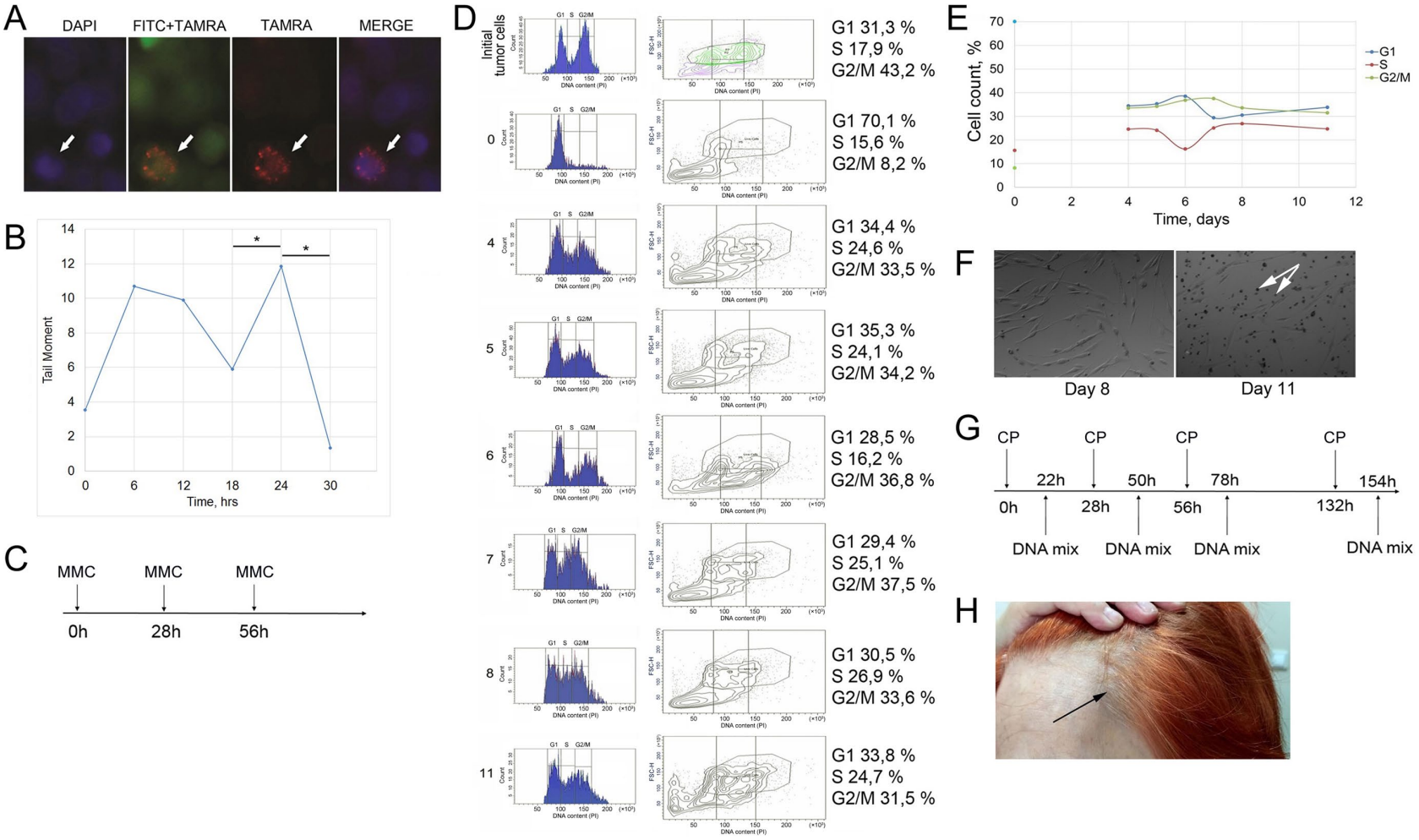

Figure 5. *Karanahan* details for patient no. 6. (A) Internalization of the TAMRA-labeled DNA probe into tumor cells. (B) The repair process in BC cells after exposure to 1 μg/mL of mitomycin C. (C) Three exposures to mitomycin C (MMC), to estimate the day of cell synchrony. (D) Estimating the day of cell synchrony after 3 exposures to mitomycin C at a dose of 1 μg/mL; percentages of cells at different phases of the cell cycle are indicated. (E) The dynamics of changes in G1, S, and G2/M cells. (F) Cells on days 8 and 11 after 3 exposures to mitomycin C; arrows point to cell debris. (G) The timeline of injections with the cross-linking cytostatic agent CP and DNA-mix. (H) The view of the temporal metastasis after 2 courses of therapy. CP indicates cyclophosphamide.

Table 5. The size of metastases in patient no. 6 before, during, and after *Karanahan*.

EXAMINATION DATA	IN THE LEFT TEMPORAL REGION	IN THE LEFT INGUINAL REGION	IN LIVER
Before first course	6.5 cm × 5.5 cm × 2.0 cm	5.5 cm × 4.5 cm × 2.0 cm	3.6 cm × 3.0 cm
Before second course	1.0 cm in diameter, flat	2.0 cm × 1.5 cm	Not available
1 month after second course	Residual fibrotic lesions	Residual fibrotic lesions	Not available
18 months after second course	No new neoplasms were revealed by MRI of head or multislice CT of thoracic and abdominal organs		

Abbreviations: CT, computed tomography; MRI, magnetic resonance imaging.

After 3 exposures to mitomycin C, the time point of cell synchronization was identified (Figure 5D). Profiling the cell cycle distribution revealed the following. On day 6 of the experiment, the number of S-phase cells decreased abruptly against the background of their prominent accumulation in G1. On day 7, the restoration of the cell cycle pattern to the regular shape was observed (Figure 5D and E). The depletion of cells from the S phase on day 6 indicates their synchronization in G1, which presumes their readiness for the synchronous exit into S phase that was successfully observed on day 7. Thus, the 6th day was considered the most suitable for the terminal treatment.

The patient was given chemotherapy with CP at a dose of 300 mg/m^2 at hours 0, 28, 56, and 132. Twenty-one days later, the patient was given a second course of joint CP and DNA-mix therapy. A total of 0.5 mg of DNA-mix was administered both into the inguinal lymph node and subcutaneous metastasis in the left temporal area 22 hours after each CP administration (Figure 5G).

The rate of change in the size of metastases in patient no. 6 is shown in Table 5. After 2 courses of therapy, metastases in the temporal and inguinal areas have reduced to anatomically undetectable state indicating complete tumor regression (Figure 5H). The dynamics of liver metastasis resorption was not followed. Neither brain imaging nor multislice computed tomography of thoracic and abdominal organs performed 18 months after the end of therapy revealed any metastatic foci in the patient's body.

Discussion

TAMRA+ TISCs in BC

A total of 34 BC specimens were analyzed. According to immunohistochemical examinations, 8 were luminal A; 11 were luminal B HER2/neu−; 11 were luminal B HER2/neu+; 1 was nonluminal HER2/neu+; and 3 were triple negative (Tables 1 and 4).

Our long-term studies indicate that in all tumor models we have worked with, the subpopulation of tumor cells capable of internalizing the TAMRA-labeled DNA probe represents the fraction of tumor-initiating stem-like cells.[58,59] In this regard, we have estimated the percentage of TAMRA+ cells in a number of tumor specimens and found it to range from 0.3 to 5.5 (Table 2).

Cancer stem cells were first isolated in 2003.[36] These cells were shown to be characterized by high surface expression of the ESA and CD44 molecule, as well as lack or low expression of CD24.[36,37] In some cases, a high activity of aldehyde dehydrogenase (ALDH), a detoxification enzyme responsible for the oxidation of intracellular aldehydes, has been demonstrated in cancer stem cells.[62] Importantly, cells with a characteristic set of markers (CD44+/CD24−/ALDH+) manifest the highest tumorigenic potential and as small as 20 of such cells are capable of providing tumor development.[63]

The content of CD44+/CD24− cancer stem cells was shown to correspond to a particular molecular subtype of BC.[38,39,64] Table 4 shows the comparative percentages of TAMRA+ cells we have determined in experiments and those of CD44+/CD24− cancer stem cells known from published data. As can be seen from the comparison, the "expected" percentage of CD44+/CD24− cancer stem cells[65] differs from the experimentally determined for TAMRA+ cells.

As is known, there is a similar discrepancy between the percentage of CD44+/CD24− cancer stem cells and that of ALDH+ cancer stem cells. It was shown that the overlap between CD44+/CD24− and ALDH+ cells ranges from 0% to ⩾60%.[62,63,66,67] This fact means that cancer stem cells represent a labile biological entity and the presence of various types of specific marker molecules most probably depends on their functional status. Our studies[58] have suggestive evidence that the ability to internalize fragments of double-stranded DNA is a feature of the most poorly differentiated cells (such as CD34+ hematopoietic stem cells, for example) and their closest progeny. A direct comparison of CD44+/CD24− cancer stem cells and TAMRA+ cells has confirmed the presumption that TAMRA+ cells are nothing else but poorly differentiated cancer stem cells: from ~10% to 30% of TAMRA+ cells were CD44+/CD24−.

It can be assumed that the TAMRA+ cells are poorly differentiated cancer stem cells that comprise both CD44+/CD24− and ALDH+ subpopulations of cancer stem cells,[38,39] as well as possibly those still unidentified.

The proliferative status of BC cells in primary cultures

Proliferative status was assessed using propidium iodide and Flow cytometry. The content of S and G2/M cells for the

luminal A subtype and the luminal B HER2/neu− subtype ranged from 3% to 50%. In luminal B HER2/neu+, 4% to 20% of cells were dividing. In nonluminal HER2/neu+ and triple-negative BC, this figure was 14% to 31%. Flow cytometry results obtained using propidium iodide were not always consistent with the Ki-67-based results (Table 4). The nuclear antigen Ki-67 is a specific marker of proliferation, and its content is 1 of the most important characteristics of the tumor phenotype, which essentially designates tumor growth rates, the risk of metastasis, the potential response to treatment, and the outcome of the cancer disease. The index of proliferative activity differs between individual tumors, being an independent factor determining the prognosis and clinical course of the disease. At Ki-67 values below 15%, the tumor is considered poorly aggressive and above 30%, the tumor is considered highly aggressive. At high Ki-67 percentages, the tumor is more likely to respond to chemotherapy. At low Ki-67, the BC will—under certain conditions—better respond to hormone therapy.

According to the propidium iodide-based Flow cytometry analysis, 57% of the tumors contained very low fraction of dividing cells (Figure 3B). To explain this generally unexpected finding, we hypothesized the following. We believe that such a pattern is due to the very strict order of cell division in tumors, which is initiated as a division of stem-like cells. These cells make up an insignificant minority of the tumor bulk and their proliferation cannot detectably affect the observed cell cycle profile. At a certain stage of tumor development, the content of dividing stem-like cells begins to exceed a certain threshold (eg, 1 cancer stem cell per 100-1000 committed cells[57]), and they "send" a humoral or whatever other signal to their committed progeny capable of proliferating to proceed to division. Once the equilibrium between cancer stem-like and committed cells is restored, the bulk of committed cells returns to the G1/G0 phase of the cell cycle again. As a result, tumors of this type exhibit an intermittent pulse growth.

From a clinical standpoint, such tumors, being in the dormant phase of the growth, are resistant to the effects of DNA-damaging cytostatics, and assessing the proliferative status of cells obtained from biopsied material is the simplest prognostic test, allowing the selection of adequate therapy. The same idea was also proposed by Vozny et al.[68]

Possible causes of DNA repair induction and proliferation activation in "dormant" primary BC cell lines

Exposure of "dormant" tumor cells to mitomycin C was found to also be followed by the characteristic pattern of DSB appearance/disappearance, similar to that in actively proliferating cells. Moreover, in 6 of 14 (43%) primary cell lines, activation of the proliferative activity after such an exposure was observed (Figure 4), suggesting mitomycin C to be somehow capable of activating this process.

At the same time, it was found that the number of DSBs detected by the "comet" assay at the "zero" point in the "dormant" tumor specimen (patient no. 5) drastically exceeds the expectations, implying no strict correlation between the proliferation and DSBs.

We hypothesized the following explanation for this phenomenon. (1) Active transcription, being typical of tumor cells, is known to be associated with a large number of transient topological breaks,[69-72] and it is these breaks that are detected at the "zero" point in G1/G0 cells. (2) In "dormant" cells treated with cross-linking cytostatics, ICLs formed in actively transcribed DNA loci interfere with the transcription complex progression and consequently induce the same repair process as in actively proliferating ones, which is associated with the nucleotide excision, which, in turn, proceeds with the formation of DSBs.[73] In this case, all transient, transcription-associated DSBs disappear, exactly as we observed at 6-hour check point, while those associated with NER begin to accumulate and are detected at further check points. Generally, this process does not affect the proliferative status, that is, "dormant" cells remain to be dormant, but there is a possibility that the ongoing NER will somehow activate the system responsible for the cell cycle arrest (which is mandatory in proliferating cells during the DNA repair) or just mimic these molecular events. As a result, on the completion of DNA repair process, the cell cycle machinery switches from the "arrested" status to the "allowed" one, and cells, with certain probability, enter the S phase, which have inevitably to end with mitosis (or apoptosis, if something goes wrong), and this point may serve as an impetus for the activation of cancer cell proliferation and accelerated tumor growth after treatment with anticancer drugs, which is a known clinical issue.[38,39,62,74]

Conclusions

We have characterized some biological peculiarities of BC specimens. The main result of this work is the strict and clear description of the requirements ("inclusion criteria") to tumor specimens, which both allow and determine the implementation and clinical validation of *Karanahan* technology. It has been established that for reliable determining the main *Karanahan* input parameters, a minimum of 4 cm^3 of tumor material is required and the tumor cells should be actively proliferating. Finally, a pilot application of *Karanahan* in clinical settings has proven to be effective in a patient with BC.

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Author Contributions

ASP performed the analysis and drafted and coordinated the article; VVK performed experiments and interpreted data; YRE performed cell cycle analysis on a flow cytometer; EVD, VSR, and GSR performed the molecular studies;

EAP, SSK, and EVL performed the analysis; AAO and ERC participated in the study design; OGB and SVS performed surgeries and worked with patients; and SSB conceived the study, participated in its design, and drafted and coordinated the article. All authors read and approved the final article.

Consent for Publication

No individual person's data included; all data are reported in an aggregate manner.

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Supplemental Material

Supplemental material for this article is available online.

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