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STUDY OF THE EFFECTS OF VARIOUS CRYOPRESERVATION METHODS ON VIABILITY OF HUMAN GASTROINTESTINAL TUMOR XENOGRAFTS IN *IN VIVO* MODELS

Background. Modern research requires access to tumor models for various types of studies on a single patient xenograft. Biobanks store tissue fragments and cell cultures for various studies, and it is necessary to form protocols for cryopreservation of various tumor fragments and create collections of biomaterial. **Material and methods.** The study was performed on tumor material from patients with malignant gastrointestinal tumors (esophagus, stomach, and colon). An experiment included 90 Balb/c Nude mice. The third generation of a PDX model was used for cryopreservation. The effectiveness of 3 protocols for cryopreservation of tumor tissue was evaluated using RPMI nutrient medium, fetal bovine serum (FBS), dimethyl sulfoxide (DMSO) and Mr. Frosty containers. 90 days after the freezing of the tumor nodes, the samples were thawed and implanted to animals; engraftment was noted, and the onset of tumor nodes in animals was recorded. At the end of the experiment, the data were analyzed statistically. **Results.** Protocol 1 was the least suitable for preserving fragments of esophageal, colon, and gastric tumors. Protocols 2 and 3 showed higher engraftment rates. The lowest engraftment rate was registered for reanimated gastric cancer PDXs. **Conclusions.** Protocols 2 and 3 with slow freezing of samples should be used for cryopreservation of human esophageal and colon cancer xenografts. Gastric cancer PDXs require other cryopreservation methods due to the low efficiency of the existing ones.

Keywords: cryopreservation, PDX, esophageal cancer, stomach cancer, colon cancer, in vivo.

Introduction. Patient-derived xenograft (PDX) models are increasingly used for various *in vivo* studies. These models reproduce the morphological and biological characteristics of the disease close to human ones [3]. Modern research requires access to biological models designed on tumor material obtained from a single patient. Such studies require a collection of biological material, such as fragments of tumor nodes, certified cell lines, and primary cell cultures [4]. This collection allows using previously generated PDX models in future studies. Its creation is based on the development and testing a tumor fragment freezing procedure and an assessment of its viability after reanimation from cryogenic freezing [10]. With patient-derived xenografts, this procedure allows the formation of a biobank of early-generation PDXs with the possibility of their thawing and re-implantation at any time depending on demand [1, 7].

The purpose of this study was to test three methods of cryopreservation of tumor material obtained from a patient and to assess the engraftment of these samples after rehabilitation.

Material and methods. *Tumor material.* The study was performed on tumor material from patients with malignant gastrointestinal tumors (esophagus, stomach, and colon). All patients gave their written informed consent for the biological material transfer. The study was approved by the ethical committee (protocol №4 from 15.02.2021).

The patients were diagnosed with:

- Esophageal cancer – squamous cell cancer T3N1M0.
- Stomach cancer – adenocarcinoma T3N1M0.
- Colon cancer – adenocarcinoma T3N0M0.

Biological material was collected immediately after excision of the tumor nodes; the tissue was placed in a DMEM nutrient medium with an antibiotic (gentamicin) and delivered to the laboratory. Prior to implantation, tumor fragments were cleaned of necrosis, blood vessels, and connective tissue and divided into fragments 1x1x1 cm.

Animals. The experiment included 90 Balb/c Nude mice (females) aged 4-5 weeks, weighing on average 23 g. The animals were kept in the SPF vivarium at the Experimental Laboratory Center, National Medical Research Centre for Oncology.

The animals were divided into groups and subgroups:

1. Esophageal cancer – 36 mice divided into 3 subgroups with different cryopreservation protocols, 12 animals each;
2. Colorectal cancer – 36 mice divided into 3 subgroups with different cryopreservation protocols, 12 animals each;
3. Stomach cancer – 18 mice divided into 3 subgroups with different cryopreservation protocols, 6 animals each.

The study was approved by the bioethical committee.

Tumor model creation. The third generation of subcutaneous PDX tumors of the human gastrointestinal tract was used for cryopreservation of biological material; the generation was obtained in previous successive inoculations and characterized by 100% transplantability. The recipient animals received anesthesia with 2 stages: introduction of the muscle relaxant xylazine (15 ml/kg of body weight of Balb/c Nude mice); introduction of general anesthesia - zoletil (67.5 ml/kg of body weight of Balb/c Nude mice). An incision was made on the side of the mouse above the rear paw, a pocket of adipose tissue was formed in the cavity of the surgical wound, and a fragment of the donor tumor was placed into it.

Cryopreservation. When the xenografts reached a volume of 400 mm^3, the tumor node was isolated from the animal body, and connective tissue, necrosis,

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and blood vessels were removed; then it was divided into fragments 3x3x3 mm and placed in a sterile cryogenic tube with a mixture for cryopreservation.

The study assessed the effectiveness of 3 protocols for cryopreservation of tumor node fragments.

1. Protocol 1. Medium for cryopreservation: 80% of RPMI 1640 medium; 10% FBS; 10% DMSO.
2. Protocol 2. Medium for cryopreservation: 90% FBS; 10% DMSO. Samples were freezed and stored in Mr.Frosty containers (Thermo Fisher) at -80°C.
3. Protocol 3. Medium for cryopreservation: 80% of RPMI 1640 medium, 10% FBS, 10% DMSO. Samples were freezed and stored in Mr.Frosty containers (Thermo Fisher) at -80°C.

Cryopreservation lasted on average for 90 days for all protocols.

Assessment of tumor engraftment after cryopreservation. 90 days after freezing of the tumor nodes, the samples were thawed in accordance with standard protocols by rapid heating in a 37°C water bath for 90-120 seconds to achieve maximum viability [8]. After reanimation, the samples were washed in a nutrient medium to remove DMSO and implanted into animals. The number of engrafted implants was noted, and the onset of tumor nodes in animals was recorded.

Statistical analysis of the data. Differences between groups were determined using parametric Student's test.

Results and discussion. We analyzed the engraftment rate of xenografts after cryopreservation. The data showed that protocol 1 was the least suitable for preserving fragments of esophageal, colon, and gastric tumors. Protocols 2 and 3 showed higher engraftment rates. The highest engraftment was observed for esophageal cancer PDXs: 41.6%; 83.3%; 83.3% for protocols 1, 2, 3, respectively. The lowest engraftment rate was registered for reanimated gastric cancer xenografts. The data on the engraftment rates are shown in Figure 1.

In addition to engraftment, we evaluated time to the growth of xenograft tumor nodes, since it can suggest the degree of cell viability after cryopreservation. The longest time to tumor growth was registered for protocol 1: the average time was 70±2.9 days for esophageal cancer, 81±1.0 days for gastric cancer, and 74±3.2 days for colon cancer. Protocols 2 and 3 led to similar results. Time to growth of xenografts of esophageal and colon cancer in protocol 2 was on average 41 days ±1.9; ±2.3, respectively, and tumor nodes of gastric cancer formed

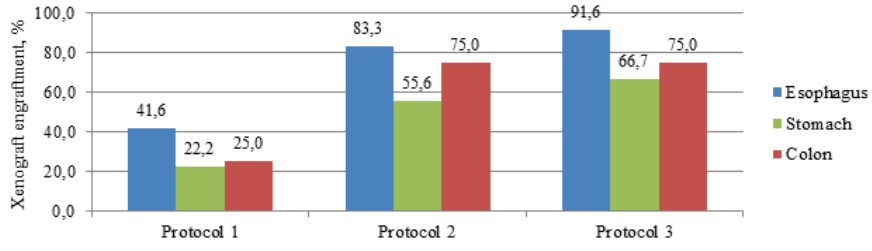

Fig. 1. Engraftment rates for xenografts of esophageal, stomach and colon cancer after cryopreservation

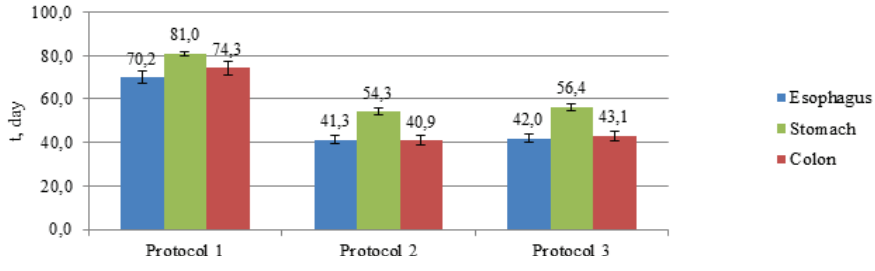

Fig. 2. Time to growth of xenografts of esophageal, stomach and colon cancer after cryopreservation

Comparison of the onset rates of esophageal, stomach and colon cancer after cryopreservation according 3 different protocols

Tumor	Protocol 1/protocol 2 p-value	Protocol 1/ protocol 3 p-value	Protocol 2/ protocol 3 p-value
Esophagus	0.002	0.002	0.84
Stomach	0.1	0.08	0.39
Colon	0.016	0.018	0.63

after 54 days ± 1.7 from the time of implantation. The growth of esophageal tumors in protocol 3 was noted at 42±1.9 days; gastric tumors on average 56±1.6 days; and colon on average 43±2.3 days from the time of implantation. The data is shown in Figure 2.

Statistical analysis of tumor formation rates showed significant differences between protocols 1 and 2, and protocols 1 and 3 in groups with xenografts of esophageal cancer and colon cancer (Table 1). The comparison of protocols 2 and 3 did not demonstrate significant differences in all groups. The results for three cryopreservation protocols did not differ significantly in the group with gastric cancer xenografts.

Discussion. Some studies report the development of various protocols for cryopreservation of tissue samples derived from animals and humans. Munroe et al. determined the advantage of DMSO as a cryoprotectant on the cells of the marine sponge Dysidea etheria in comparison with other tested substances [6]. Faltus et al. studied the thermal properties of various cryoprotectants; as a result, they also recommended DMSO as a cryoprotective agent for freezing cell suspensions [2]. Based on these studies, we chose DMSO as a cryoprotectant for freezing PDX fragments. In mixtures, it ensured the preservation of cell viability after thawing of the samples. However, in protocol 1 (freezing in a mixture of 80% RPMI 1640 culture medium, 10% FBS, and 10% DMSO) showed the lowest engraftment rate and longer time to tumor growth, compared with the other two protocols. Probably, this difference was due to the higher freezing rate in the first case than in the other two. The rate of freezing of samples directly affects their viability after reanimation, since slow freezing is accompanied by the outflow of intracellular fluid preventing the formation of ice in the cell [5]. In our study, the necessary rate was provided by the Mr. Frosty freezing container filled with isopropyl alcohol, it provided a freezing rate close to -1°C/min. However, all protocols demonstrated poor engraftment for human gastric cancer samples. According to Yan et al. creating a comprehensive collection of gastric cancer biological material, tumor fragments were subjected to instant freezing in order to successfully preserve

cell viability, which prevented the formation of ice crystals that injured cell walls [9]. Apparently, slow freezing, suitable for esophageal and colon cancer, was not applicable to the stomach tumor tissue. We will take it into account in the further development of cryopreservation protocols.

Conclusions. Protocols 2 and 3 with slow freezing of samples should be used for cryopreservation of human esophageal and colon cancer xenografts. Gastric cancer samples require other cryopreservation methods due to the low efficiency of the existing ones.

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GENETIC AND IMMUNOLOGICAL MARKERS OF THE FORMATION OF METABOLIC SYNDROME IN SCHOOL CHILDREN (ON THE EXAMPLE OF THE PERM REGION)

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The problem of the formation of metabolic syndrome in children is becoming more and more urgent every year, which is associated with excess nutrition, physical inactivity, increased psycho-emotional stress, therefore, timely identification of immune and genetic markers of predisposition to the development of this pathology will allow identifying possible health risks at an early stage and preventing their implementation in adulthood. **The aim of the study:** To evaluate the indicators of immune status and genetic polymorphism of candidate genes as markers of the development of metabolic syndrome in school children (on the example of a secondary school in Perm). **Materials and methods.** The study involved 214 school-age children. Three groups were formed, ranked according to the body mass index criterion: observation group1 with metabolic syndrome (BMI SDS >2.0), observation group2 with excess body weight (BMI SDS >1.0 <2), comparison group – absence of excess body weight (BMI SDS <1.0). The evaluation of immune (IL 1B, IL 4, CD19+), neuroregulatory (leptin), metabolic (glucose, HDL, triglycerides), genetic (ADRB rs1042413, PPARA rs4253778) indicators was carried out. **Results and discussion.** It was found that the group of children with metabolic syndrome and excess body weight in relation to the comparison group was characterized by an increase in CD19+ expression by 1.3 times, a decrease in the content of anti-inflammatory cytokine IL4 by 1.5 times, overexpression of pro-inflammatory cytokines (IL1b by 1.9 times), leptin by 2.0 times, an imbalance of lipid-carbohydrate metabolism (reduction of HDL by 7%, against the background of an increase in triglyceride levels by 17% and glucose levels by 8%), significant changes in the frequencies of genotypes associated with metabolic syndrome (increased frequency by 2.7 times of the typical AA genotype of the ADRB2 gene rs1042713, OR=3.79 CI:1.25-11.47; p<0.05, as well as by 4.6 times of the variant CC genotype of the PPARA gene rs4253778 OR=5.00; CI:0.97-25.89; p<0.05). **Conclusion.** Candidate immunological (CD19+, IL 1b, IL4) and genetic (ADRB2 rs1042713, PPARA rs4253778) markers are recommended to be used as indicators for identifying early signs of metabolic syndrome in school-age children living in the Perm region.

Keywords: metabolic syndrome, body mass index, PPARG gene, ADRB2 gene, cytokines, CD, schoolchildren.

Introduction. The peculiarities of the development of the child's body, as well as bad habits, sedentary lifestyle, excess nutrition, genetic predisposition, increased emotional stress lead to the formation of metabolic syndrome in children become risk factors for the development of cardiovascular diseases in adulthood. If earlier the diagnosis of metabolic syndrome was applicable only to the adult