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Methodological aspects of creation of patient-derived tumor xenografts

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Abstract

High rates of cancer incidence and mortality from malignant neoplasms remains an urgent health problem. The development of the most effective therapeutic algorithms is required to improve the survival of cancer patients. An important condition for the discovery of new anticancer drugs and their translation into clinical practice involves the ability to model tumor growth, reproduce the characteristics of human disease, and evaluate measurable effects of pharmacological substances in laboratory facilities. Xenograft models established by direct implantation of fresh tumor tissue samples from individual patients into immunodeficient mice are considered suitable for both preclinical trials and for solving fundamental problems in oncology. The review highlights the significance of patient-derived xenograft models as a platform with high predictive value and the prerequisites that make them the preferred tool for research in cancer biology. The most important methodological aspects in the creation of these models are considered. Methods for obtaining and preparing biological tumor samples for xenotransplantation are discussed. The significance of the immune status, as well as the phenotypic and genetic characteristics of recipient animals, is described. The article presents the limitations of animal models associated with their immunodeficiency status and ways to overcome them. The principles for choosing xenotransplantation sites (heterotopic and orthotopic) and their advantages and disadvantages are discussed. In conclusion, we emphasize the need to continue the work on optimizing PDX (Patient-Derived Xenograft) models to overcome their limitations and to obtain the most reliable and valuable research results in oncology.

Keywords: oncology, cancer, xenografts, PDX, immunodeficient mice.

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Malignant neoplasms represent one of the major causes of death and remain an urgent public health problem [1–3]. Numerous research programs aimed at studying and improving progress rate in the prevention, diagnosis, and treatment of cancer have contributed to significant success [4]. Nevertheless, the development of the most effective therapeutic algorithms is still much needed and remains a prerequisite for improving the survival rates of cancer patients.

New research methods and tools such as clinical bioinformatics, disease biomarker studies, and model experiments play an important role in the process of drug development [5–7].

A fundamental condition for the discovery of candidate substances with a probable antitumor effect and their advancement into clinical practice is the ability to simulate tumor growth, demonstrate the characteristics of a human disease, and evaluate measurable effects of anticancer drugs in laboratory facilities [8].

A recent study revealed that reinvestment of the pharmaceutical industry in the field of oncology was significantly lower than in other therapeutic areas [8]. About 95% of potentially effective antineoplastic substances that successfully passed phase I clinical trials and demonstrated good tolerance did not show efficacy in subsequent phases, and therefore were not registered. According to a number of experts, the most common reasons of the drug inability to reveal its clinical efficacy is the absence of validated preclinical models or an incompletely established relationship between a specific therapeutic target and a disease [4, 9, 10].

Traditional drug screening methods were developed at the USA National Cancer Institute (NCI) in the 1970s. They suggested testing antitumor agents *in vitro* and *in vivo* using a panel of hu-

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man cancer cell lines NCI-60 [11, 12]. Cancer cells were obtained from patients with malignant tumors and adapted to grow on plastic under artificial culture conditions. These so-called immortalized cell lines, although convenient and easy to use, have significant limitations expressed in serious and irreversible changes in biological characteristics: including changes in the properties of growth and invasion, as well as the loss of certain cell populations, which can be described as the absence of morphological and molecular genetic heterogeneity [8, 9].

Attempts to bypass these barriers have led to the development of xenogenic models obtained by direct implantation of fresh tumoral samples from individuals by immunodeficient mice (PDX, patient-derived xenograft) [9, 13]. In numerous studies, they were considered as the best predictors of therapeutic response, since they were able to preserve the heterogeneity and molecular characteristics of the original tumor at early passages and were considered more suitable tools for both preclinical trials and problem solving in many areas in basic science [13, 14].

Therefore, patient-derived xenograft models are increasingly being used for drug development. For example, from the works of the National Institute of Oncology, there is a tendency to transfer from the use of NCI-60 panels of cell lines to the use of PDX. This is in line with the works of the Novartis Institute for Biomedical Research with clinical significance attaining 90% [15, 16]. These results indicate favorable prospects for the application of PDX in academic and applied oncology [17, 18].

Obtaining biological samples for xenotransplantation: The PDX creation procedure is of a standard nature and follows the general algorithm, although some scientific groups mention the development of separate approaches [13, 19, 20]. PDX models can be created by transplanting tumoral tissue obtained during surgery or biopsy, from a patient to immunodeficient mice [21–23]. Some studies have also used samples of ascitic fluid or pleural effusion [24, 25].

The resulting tumor tissues are washed and stored in cell culture media with antibiotics [26]. In order to maintain maximum viability, they need to be transplanted into recipient animals as soon as possible. Long-term ischemia is known to be associated with a lower degree of the biological material engraftment [27]. If the procedure could not be performed immediately, they should be stored in a refrigerator immediately to minimize tissue metabolism.

Tumoral tissue is implanted in the form of fragments or cell suspensions, either isolated or in combination with Matrigel, fibroblasts, or mesenchymal stem cells [28]. The injection of a cell suspension obtained through mechanical or chemical disaggregation of a tissue sample has been elaborated in some studies [29, 30]. However, it is considered a less preferable option, since it is characterized by a lower intensity of engraftment of tumoral tissue, which can probably be associated with anoikis (a form of apoptosis that occurs in response to the loss of connection with the matrix or due to the cell detachment from neighboring cells) [31]. Tumor fragments are more often surgically implanted into a heterotopic (subcutaneous) site, but a histologically corresponding organ is used to create orthotopic models (patient-derived orthotopic xenograft, PDOX) [4, 32, 33].

It usually takes about 3–6 months to produce the first-generation xenograft; its growth rate depends on the individual characteristics of the tumor [27, 34]. When the size of tumor nodes reaches 500–1000 mm^3, they are isolated, fragmented and used to create the next generation of PDX, as well as for histological, immunohistochemical, and molecular genetic studies. A number of fragments are also cryopreserved for repeated implantation at any convenient time, when necessary [13, 27] (Fig. 1).

Several studies revealed that the success of PDX creation was associated with tumor aggression, and patients whose tumors were successfully transformed in the PDX model had worse prognosis than those who did not succeed in generating PDX [20, 28].

Immune status of animals-recipients of tumor material: The immunodeficient status of recipient animals is a prerequisite for preventing the rejection of tumor material of another biological species, and therefore a large number of mouse strains have been developed, characterized by varying degrees of their immune system dysfunction [35].

Several types of immunodeficient mice can be used to create xenograft models, namely thymus-deprived nude mice (Nude), SCID, NOD-SCID, Rag2, NSG, NOG, which all differ in the degree of immunosuppression in relation to the functions of immune cells, and these characteristics should be evaluated according to the study. Nude does not develop T-cells, since thymus development is inhibited by mutations in the *Foxn1* gene [35–37]. SCID mice do not have T- and B-cells due to a mutation in the *Prkdc* gene; in Rag2 knockout mice, the differentiation of B- and T-cells is blocked due to gene *Rag2* eviction even more completely than in the case of a natural mutation in the *Prkdc* gene [36, 38]. NOD/SCID mice do not exhibit the functions of T-cells, B-cells, and natural

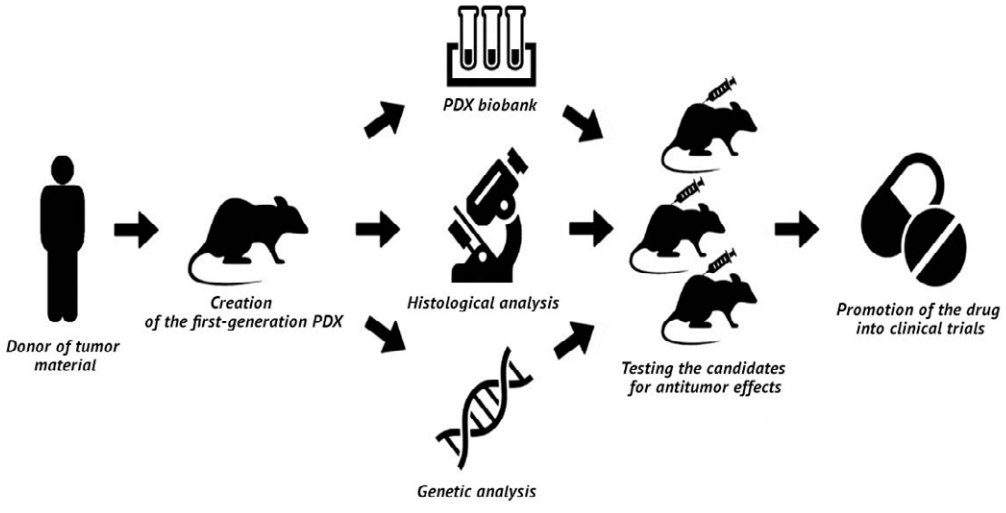

Fig. 1. Diagram of the general procedure for creating and using PDX models.

killer (NK) cells [35, 36]. NSG and NOG have lost the functions of T-cells, B-cells, and NK-cells, as in NOD/SCID mice, as well as have a mutation in the gene for the γ chain of the IL2 receptor. The latter therefore have increased transplantable characteristics and are able to engraft almost all types of human cancer [39, 40].

Recently, by removing *Foxn1* using the CRISPR/Cas9 system, a series of NOD/SCID/IL2rg–/–nude mice named NSIN, were obtained, which showed an even more profound immunodeficiency than mice of other strains, and may be more suitable for oncological studies with low engraftment efficiency [4]. Currently, work on the creation of animals with a high degree of immunodeficiency is being conducted by many groups, and the list of new lines of mice with the required properties is constantly being updated [41, 42]

The higher the degree of immunosuppression, the greater the probability of success and the rate of formation of the PDX model: however, when using animals with the most pronounced defects of the immune system, problems can arise due to the activation of viruses of human origin, such as the Epstein–Barr virus, which entails the development of human lymphoma. Thus, it becomes expedient to test the models obtained for the absence of lymphoma [27, 43].

Choosing a site for transplantation: The subcutaneous (heterotopic) site is most commonly used to transplant tumor material for PDX production. This site is simpler to manipulate for PDX creation, and tissue damage can be minimized: thus, ensuring an easy and painless recovery of the animal after surgery. In addition, an important advantage of this method consists in the direct assessment of tumor engraftment and growth without the use of special equipment for imaging or control laparotomy. However, a subcutaneous xenograft grows in a microenvironment inappropriate to the original tumor and, as a rule, is not capable of reproducing the metastatic process [27, 44].

In order to overcome these disadvantages, an orthotopic transplantation site corresponding to the site of the primary tumor should be employed. This procedure is technically more complex, time-consuming, and requires ultrasound or diagnostic laparotomy to confirm the presence of tumors inside the mouse. However, it is beneficial in that it reproduces a better "natural" surrounding of human tumors. Orthotopic implantation can increase the frequency of metastases during xenograft growth and should be considered a priority when tumor metastasis is the center of research [4, 34, 44].

According to a study by Y. Koga and A. Ochiai (2019), the creation of subcutaneous PDX is the most common procedure (80%) [15]. Orthotopic implantation was chosen as more preferable for several types of cancer, including primary or metastatic brain tumor [45, 46], breast cancer [47, 48], and renal cancer [49]. Although these models mimic cancer metastases and serve as important models for basic and applied research, subcutaneous xenografts are usually used in preclinical trials, since the PDX-skin complex enables the easy evaluation of drug efficacy compared to PDOX models [15].

Problems of creating PDX models and their solutions: Despite the fact that PDX models provide an excellent opportunity to increase the translational potential of biomedical research in the field of oncology and have several advantages over traditional xenograft cell line models, they, like any other platforms for preclinical trials, have several significant limitations.

The most common problems in PDX production are associated with insufficient engraftment efficiency of the primary tumor material. To overcome this disadvantage, in addition to obvious solutions, such as the use of animals with the most pronounced forms of immunodeficiency as recipients, optimized xenotransplantation methods were proposed, using sites characterized by a well-developed vascular network. In particular, there is an approach where implantation into the subrenal capsule is performed to increase the engraftment success regardless of the tumor origin [50–52]. The use of this technique provides an abundant supply of nutrients, hormones, growth factors, and oxygen for the transplanted tissues even before the establishment of the graft vascularization [50].

When comparing the procedures for xenotransplantation of malignant tissues of the human prostate gland into the subrenal capsule of immunodeficient mice, into the subcutaneous and orthotopic sites, the best engraftment results obtained corresponded to the renal site [53, 54]. In addition, a greater vascularization of the renal site contributes to the preservation of the heterogeneity of the initial sample of the primary tumor, thereby preventing the selection of cell populations that are resistant to oxygen starvation associated with the initial phases of the process during transplantation into the subcutaneous site. This technique has been successfully used both to obtain PDX for prostate cancer [55] and to create PDX for non-small-cell lung cancer [56], cervical cancer [52], and ovarian cancer [57].

Also J. Lee et al. (2019) proposed an original author's technique for creating a PDX model of glioblastoma by intravitreal injection, which, on the one hand, promotes the formation of a tumor in the microenvironment that mimics the brain, and on the other hand, provides improved visibility and control of growth dynamics [58]

Nevertheless, advancements are been made to improve the efficiency of the procedure for xenotransplantation of human tumor fragments, another problem is the unequal possibility of obtaining PDX representing various types of tumors. For example, a previous study revealed that the graft survival rate is highest for malignant melanoma and colorectal cancer (80% and more), while it is only about 30% for breast cancer [20]. Thus, a situation arises in which the availability of the model is largely determined by the rate of the tumor material engraftment, rather than its clinical morbidity.

The solution to this problem is the creation of biobanks specialized in storing and providing detailed annotated PDXs, which enables to find and acquire a model with the necessary biological characteristics. Institutions such as the National Institute of Oncology (USA), CrownBio (China), the Innovative MODels Initiative consortium (France), and Novartis Institute for Biomedical Research (USA, China, Switzerland, Singapore) possess extensive collections of xenografts obtained from patients [59].

A separate problem is the impossibility of obtaining a tissue sample from a patient using a surgical procedure for unresectable tumors, which may entail a situation associated with an unbalanced representation of various types/subtypes of tumors in repositories.

In order to overcome these limitations, attempts have been made to create xenograft models by injecting circulating tumor cells isolated from blood samples of humans into immunodeficient mice [60, 61]. It is believed that circulating tumor cells represent a mixed population of cancer cells, and the isolation and inoculation of these cells in immunodeficient animals lead to the creation of a model that preserves tumor heterogeneity in patients [60, 61]. Small-cell lung cancer xenografts obtained from circulating tumor cells have significant genomic similarities with actual tumors and reflect their response to chemotherapy [62].

In addition to the above difficulties associated with the creation of models of some types/subtypes of tumors, the need to spend significant resources such as time, finances, and labor costs also constitutes a problem. In this regard, increase in the efficiency of the procedure for creating PDX models and obtaining a sufficient amount of tumor tissue for further xenotransplantations in order to conduct translational studies is one of the priority tasks [30, 63].

Z. Liu et al. (2020) proposed an original technique for working with xenografts, which reduces the number of tumor-bearing animals, as well as minimizes the time spent. In the course of the work, a series of sequential incomplete resections of subcutaneous tumor nodes were performed, allowing the remaining 5–10% of the xenograft volume to continue to grow in the same animal. In addition, tumor nodes resulting from incomplete resection grew 26–60% faster than xenografts of the same tumor that did not undergo incomplete resection [63].

One of the key aspects of creating PDX is the need to use immunodeficient strains of mice. On one hand, this is a prerequisite for the engraftment of human tumors, and on the other hand, the weakened immunity makes it impossible to model and study immune responses. For this reason, xenogeneic tumor models are of limited use in screening immunotherapeutic drugs such as vaccines and immunomodulators, as well as drugs that activate the antitumor response of the immune system.

At the same time, recent advances in oncology highlight the importance of the immune system in the progression and treatment of tumors [64–66]. The solution in this situation can be the creation of humanized PDX models by injecting human immune system cells into mice, thereby favoring both the conduction of fundamental studies of the interaction of immunity and tumors and preclinical studies of drugs that activate the antitumor response of the immune system [66–68].

One of the possible options for creating humanized mice involves the transplantation of peripheral blood mononuclear cells or tumor-infiltrating lymphocytes into immunodeficient mice [69]. These procedures are known to induce graft-versus-host reactions 2–5 weeks after injection and limit the useful study time. Another method consists in the transplantation of CD34-positive human hematopoietic stem cells alone or in combination with additional human immune tissues (such as human thymus tissue) into immunodeficient mice [69].

Hematopoietic stem cell transplantation results in a more complete imitation of the human hematopoiesis as they give rise to various human blood cell lines in mice. Thus, the next generation PDX models created using humanized mice, although expensive, will overcome the limitations associated with the immunodeficiency status of traditional xenogeneic models.

Conclusion. Over the past few years, the PDX model has gained the status of a platform with high predictive value compared to conventional xenograft cell line models. Literature demonstrates that PDX models are capable of maintaining molecular and genetic heterogeneity in human tumors. Preclinical trials in mice can reduce the risk of clinical trials in humans, as well as accelerate treatment prioritization by allowing several treatment regimens to be tested in parallel for selected patient groups. However, there is still a lot of work to be done to address a number challenges to make this approach more productive. Many research teams continue to work actively to optimize PDX models in order to overcome their shortcomings, thereby obtaining valuable research results in the field of oncology.

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