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Natural and Chemotherapy-Induced Clonal Evolution of Tumors

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Abstract—Evolution and natural selection of tumoral clones in the process of transformation and the following carcinogenesis can be called natural clonal evolution. Its main driving factors are internal: genetic instability initiated by driver mutations and microenvironment, which enables selective pressure while forming the environment for cell transformation and their survival. We present our overview of contemporary research dealing with mechanisms of carcinogenesis in different localizations from precancerous pathologies to metastasis and relapse. It shows that natural clonal evolution establishes intratumoral heterogeneity and enables tumor progression. Tumors of monoclonal origin are of low-level intratumoral heterogeneity in the initial stages, and this increases with the size of the tumor. Tumors of polyclonal origin are of extremely high-level intratumoral heterogeneity in the initial stages and become more homogeneous when larger due to clonal expansion. In cases of chemotherapy-induced clonal evolution of a tumor, chemotherapy becomes the leading factor in treatment. The latest research shows that the impact of chemotherapy can radically increase the speed of clonal evolution and lead to new malignant and resistant clones that cause tumor metastasis. Another option of chemotherapy-induced clonal evolution is formation of a new dominant clone from a clone that was minor in the initial tumor and obtained free space due to elimination of sensitive clones by chemotherapy. As a result, in ~20% of cases, chemotherapy can stimulate metastasis and relapse of tumors due to clonal evolution. The conclusion of the overview formulates approaches to tumor treatment based on clonal evolution: in particular, precision therapy, prediction of metastasis stimulation in patients treated with chemotherapy, methods of genetic evaluation of chemotherapy efficiency and clonal-oriented treatment, and approaches to manipulating the clonal evolution of tumors are presented.

DOI: 10.1134/S0006297917040022

Keywords: clonal evolution, tumor, chemotherapy, mutation, metastasis

The theory of evolution of C. Darwin was developed and applied for determining ways and patterns of the evolutionary process in speciation. Later it turned out to be applicable to other biological systems, including processes of malignant transformation. According to the theory of clonal evolution of tumors formulated by P. Nowell in 1976, mutability is a resource for development of new tumor clones, and natural selection is a basis for survival of adapted aggressive clones of tumor cells [1]. Generation of intratumoral diversity or intratumoral heterogeneity, which is a feature of most tumors, is a direct result of clonal evolution of a tumor [2-6]. According to the reference literature, there are two main hypotheses on the origin of heterogeneity in tumor cells: different subclones of tumor cells originate from different tissue stem cells, and each has its transformation trend (*polyclonal concept*); different clones of tumor cells develop from the

initial clone due to various genetic and/or epigenetic changes in the process of evolution (*monoclonal concept*) [7]. At the moment, each of these concepts is studied and has its supporters.

Intratumoral heterogeneity as a result of clonal evolution explains certain features of tumor development: the presence of tumor clones with individual set of features (for example, variants of mutation are not identically distributed in tumor cells); coexistence of morphologically different structures in the tumor; presence of neutral relations between tumor clones (with no visible phenotypical consequences); development of malignant cells resistant to medication treatment; and, what is more important, different response to treatment by tumors [8, 9].

Evolution and natural selection of tumor clones during tumor development and the following carcinogenesis may be called *natural clonal evolution*. New generations of malignant cells originating from the initial clone or from

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a multitude of stem cells, which are transformed into tumor stem cells, accumulate different molecular and genetic changes [10]. What is important is natural clonal evolution takes place due to internal mechanisms, and it is driven by driver mutations (occurring due to failures of reparation, replication, effect of carcinogens, and more), genetic instability, and mostly factors of the microenvironment that form the environment for cell transformation and their survival. Formation of so-called spatial intratumoral heterogeneity in a tumor is a consequence of natural clonal evolution [11]. In cases of spatial intratumoral heterogeneity, morphologically similar tumor cells may develop various morphological structures within a tumor and represent different populations, some of which may obtain selective advantage and lead to tumor progression. Acquisition of new genetic defects by cell populations may also conduce to them gaining competitive advantage in selective pressure of factors of the stromal microenvironment [12]. This advantage may be increased speed of subpopulation growth, gained ability to inhabit new locations (invasion and metastasis), and avoiding the effect of antitumor medication and the immune system [2, 13, 14]. As a result of clonal expansion cycles, clones may reproduce and have specific structure and numerical chromosome aberrations that will be dominant in a tumor [15, 16]. It is still difficult to answer how minor clones are pushed out by the dominant one. Nobody has studied the mechanisms of clone competition yet. This problem remains unsolved, and for now we may deem various versions acceptable. Mechanical substitution of minor clones is possible due to invasion and more rapid growth of the dominant clone. Tumor growth is followed by increasing pressure on the structures of the extracellular matrix; on this, the tissue microenvironment tries to contain its functional and anatomic entirety, while increasing interstitial pressure on the tumor cells [3]. The dominant clone may form a tumor blood circulatory system according to its needs [8], take nutrition, and limit access of minor clones to oxygen. In cases of tumor hypoxia, it may be a limiting factor for minor clones. Cells of substituted minor clones struggle to leave the tumor and to survive in the aggressive environment while resisting powerful antagonistic factors of the microenvironment. It is not a coincidence that cells entering the blood flow throughout the life of a tumor die in the blood flow in most cases. To invasively grow into adjacent tissue, tumor clones also need considerable changes, which they may not be able to make. Expression of intercellular adhesion molecules changes considerably. Epithelium and mesenchymal transition is required for single amoeboid or mesenchymal cell migration. Collective invasion requires coordinated and differentiated changes in the subpopulation of tumor cells together with forming of an invasion front [3].

The occurring genetic changes in tumor cells do not always lead to functional consequences, as some of them are neutral. In this regard, it is important to consider the concept of initial and secondary driver mutations and passenger mutations, i.e. mutations that increase adaptation of tumor cells, and neutral or negative defects, respectively. A good illustration of this is an image of driver mutations as a tree trunk and big branches, and passenger mutations as small branches and leaves [4, 13, 17, 18].

In contrast to natural clonal evolution, which takes place due to internal factors, in *chemotherapy-induced clonal evolution* of a tumor during treatment, chemotherapy is a driving factor. On one hand, chemomedication, possessing mutagen effect and affecting tumor cells, may directly lead to development of genetic defects and new tumor clones [19, 20]. On the other hand, chemomedication may destroy dominant tumor clones, clearing the space for clonal expansion of minor resistant clones [21]. Chemotherapy can be regarded as the driving factor of the evolution process in tumors. Chemotherapy-induced clonal evolution results in temporary intratumoral heterogeneity, and there are few studies that have analyzed clonal evolution after chemotherapy [11]. First, research in this direction is urgent for detecting mechanisms of forming resistance to treatment, mechanisms of tumor progression, developing new predictive markers, and identifying new targets for target therapy. It is believed that target therapy blocks growth of tumor cells by interference into the mechanism of activity in certain target molecules necessary for carcinogenesis and growth of tumor (for example, *EGFR*, *KRAS*, *HER2/new*, *VEGF*, *TERT*, *ALK*, *ESR1*, and many others), but not simply prevents cells (including tumor cells) from reproducing as traditional (conventional, cytostatic) chemotherapy. According to Hanahan and Weinberg, target therapy may be used for all 10 signs of malignancy [8].

NATURAL CLONAL EVOLUTION OF A TUMOR AND SPATIAL INTRATUMORAL HETEROGENEITY

Natural clonal evolution is initiated by genetic defects in a stem cell, and it transforms it into tumor-inducing cells. Such tumor-inducing cells have high proliferative potential and the ability to develop due to genomic instability, new functionally relevant mutations, and evolutionarily new subclones [22]. Much oncologic research is dedicated to studying natural clonal evolution of tumors in different localizations and to spatial intratumoral heterogeneity [4, 5, 13, 23-28]. In cases of primary myelofibrosis (PMF), a tumoral bone marrow disease, it was shown that the microenvironment participates in natural clonal evolution. It was followed by post-primary inflammation with changes in the stroma of bone marrow and pathological production of cytokines [29]. It is important to note that the concentration of proinflammatory cytokines in blood in cases of PMF increases,

which is followed by symptoms of tumor intoxication [30], and long proliferation of a tumor clone associated with inflammation leads to additional mutations and higher degree of malignization, which leads to blast crisis of PMF [31]. As previously shown by Tefferi et al. [32], an increase in the level of IL-8, IL-10, and IL-15 interleukins and in expression of receptors of IL-2 leads to low overall survival and survival before blast transformation, which can be connected with increasing pace of clonal evolution.

Cervical cancer is also a good model for studying natural clonal evolution and spatial intratumoral heterogeneity for several reasons: first, good accessibility; second, the etiological reason for cervical cancer development is well known: infecting of cervical epithelium with human papilloma virus; third, pre-tumoral pathologies of the cervix uteri are well known. Meta-analysis with 293 samples of squamous cell carcinoma showed that the most frequent chromosomal changes are 3q amplifications, deletions on 3p, and deletions on 11q chromosome regions. At the same time, in the pre-tumor pathology (cervical intraepithelial neoplasia, CIN), copy number aberrations (CNA) are very rare [33]. Another study using the FISH method included 168 women with CIN II-III showed an increase in frequency of amplification in the 3q26, 5p15, and 20q13 loci with increasing severity of impairment in the cervix uteri [34].

Determination of *KRAS* and *TP53* somatic mutations in colorectal cancer showed high frequency and variety of mutations at early stages of cancer development and their decrease at later stages, which may point to expansion of dominant clones during the late stages of disease. At the same time, the frequency of 5q and 18q allelic deletions remains stable throughout tumor growth [35].

Natural clonal evolution in prostate cancer at stages 3 to 4 was studied using laser microdissection in samples obtained from radical prostatectomy [36]. *TMPRSS:ERG* gene fusion and some other genetic and epigenetic features that prove general clonal origin of malignant cells were detected in all samples. Thus, cells of progressing prostate cancer (G4) originated from cells of the preceding cancer stage (G3) and had common precursors, and the studied *TMPRSS:ERG* genotype was a feature of aggressive prostate cancer.

To study different patterns of genetic regrouping connected with growth and development of a tumor, Campbell et al. sequenced 13 primary adenocarcinomas of pancreas and the respective metastases [37]. For studying clonal evolution, clonal structure of metastases, primary tumor, and phylogenetic relations between the primary tumor and metastases, Next-Generation Sequencing (NGS) DNA sequencing technology was used, which enabled not only annotating the genomic alterations, but also studying the clonal relations between metastases. It was found that pancreatic cancer acquires alterations that point to telomere dysfunctions and abnormal cell cycle

and dysregulation of the transition from G1-to-S-phase under intact G2–M transition. Even though it is expected, for early stages of cancer development this genomic instability remains and, under further development of neoplasm, parallel or convergent evolution of various metastases occurs. Genetic heterogeneity exists among metastasis-inducing clones as well, but after seeding of the latter not just a driver mutation is needed for a metastasis to occur. It occurred in the primary tumor. The authors showed that genomic instability determined multiple intratumoral heterogeneity of tumoral clones, including ones responsible for metastases [37]. Another study used NGS in metastatic pancreatic cancer to show that the metastases were insignificantly different from the primary tumor in terms of unique mutations [38].

In the case of esophageal adenocarcinoma, an increase in chromosome instability in the transition from pre-cancer state of the patient to malignant tumor was shown [39].

Breast cancer is believed to be one of the most heterogeneous forms of cancer. It was shown that tumoral clones of breast cancer can be characterized by specific structural chromosome and numerical aberrations, the presence of pseudodiploid and aneuploid cell populations that developed because of clonal expansion cycles [15, 16].

A study by Nik-Zainal et al. included genome sequencing of 21 breast tumors and their genealogy based on bioinformatic analysis of the genetic data pool. It was shown that driver mutations are initiating in terms of tumorigenesis, and they precede chromosome instability. Most of the time of breast cancer development is connected with generating intratumoral diversity [14].

Many authors have highlighted a considerable degree of intratumoral heterogeneity in terms of ploidy, chromosome aberrations, and gene mutations in breast cancer [40]. These authors also confirmed clonal evolution of breast cancer tumor: for triple-negative and ER-positive breast cancer [41]. Studies of single mutations for triple-negative and ER-positive breast cancer showed there were not two genetically identical tumor cells. There were a huge number of subclonal and newly formed mutations observed in patients. These data indicate that point mutations gradually developed for a long period, creating vast clonal diversity [42]. Intratumoral morphological heterogeneity of invasive ductal breast cancer was recently described by Russian researchers [43, 44]. They detected five types of morphological structures in the most widespread form of breast cancer, invasive ductal carcinoma, whose cells are different in the pattern of adhesion gene expression and medication resistance.

Thus, natural clonal evolution creates intratumoral heterogeneity and enables tumor progression. The main driving factors are internal: genetic instability initiated by driver mutations and microenvironment, which enables natural selection. The same localizations may be
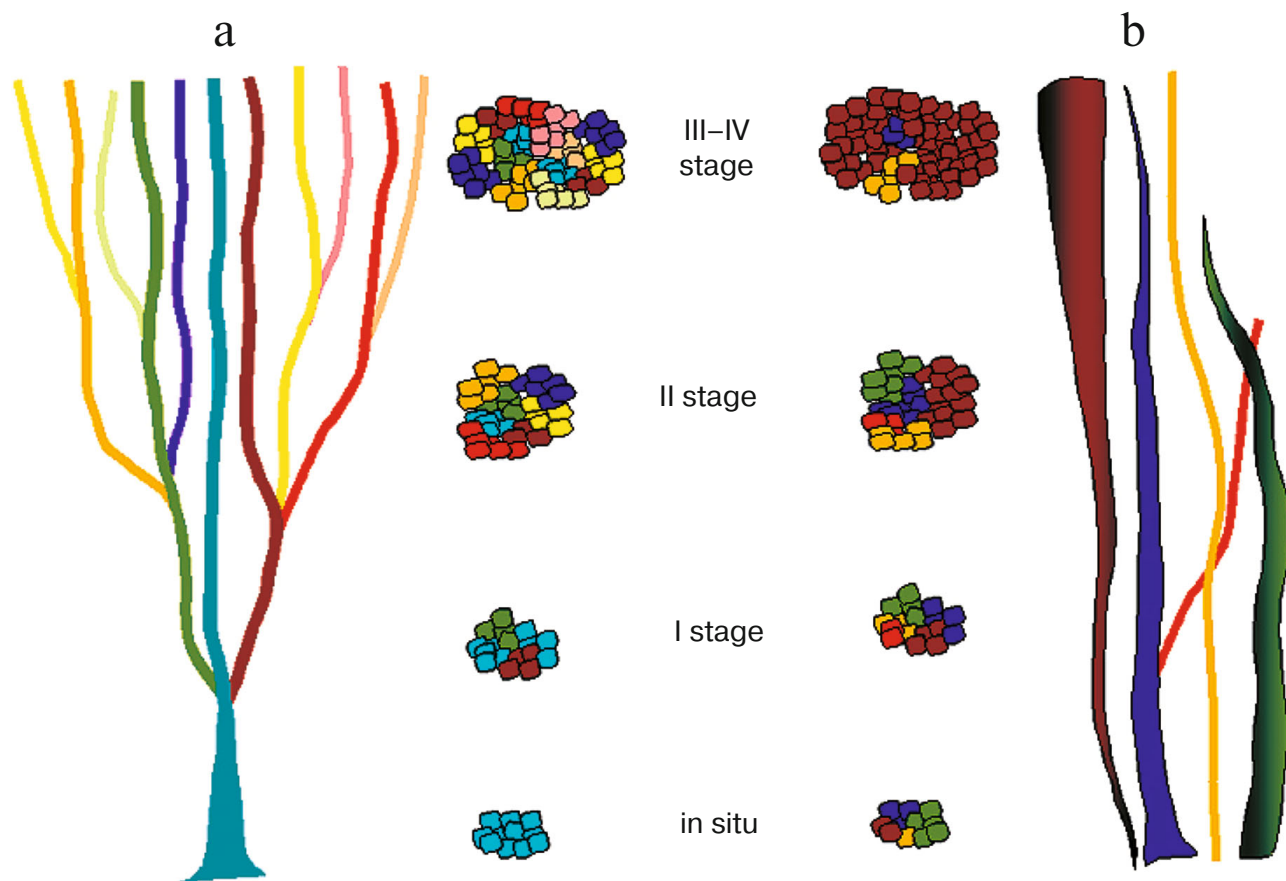
Fig. 1. Clonal evolution in tumors of monoclonal and polyclonal origin. a) Monoclonal origin of tumor (clonal diversity increases with the size of tumor); b) polyclonal origin of tumor (clonal diversity decreases with the growth of tumor). Different colors show different clones of the tumor; thickness of the lines correlates with presence of a clone in the tumor.

described by two models of natural clonal evolution (Fig. 1).

In the case of monoclonal development of a tumor, significant clonal diversity is gradually formed via divergence of new clones due to primary clones changing. Although primary clones may die in divergence of clones, such cases do not see primary driver mutations lost — they are passed on to the next generations. While diverging, they are joined by post-primary drivers and passenger mutations, which forms new clones. When the NGS method is used to study the stages of natural clonal evolution of a tumor in a particular patient, DNA of many parts of the same tumor is sequenced (dozens or even up to hundreds of tumor parts are used) individually. Primary driver mutations are determined — those are present in all studied parts of a tumor; secondary drivers are not present in all parts, and passenger mutations may be encountered only in certain parts. This method enables development of the clonal evolution tree of life. Polyclonal origin sees cells with multiple changes formed at the earliest stages, and those changes can remain or be eliminated. Tumors are differentiated accordingly: in the first case, tumors are of

low-level intratumoral heterogeneity in the initial stages, and it increases with the size of the tumor (Fig. 1a); in the second case, tumors are of extremely high-level intratumoral heterogeneity in the initial stages and become more homogeneous when larger due to clonal expansion (Fig. 1b). Clonal expansion and formation of a dominant clone in tumors of polyclonal origin occur at the later stages and are followed by a decrease in clonal diversity due to substitution of minor clones. Such tumors should respond to treatment better because of their homogeneity. In a real situation, divergence of clones and formation of dominant clones with substitution of minor clones in a tumor may occur simultaneously. Our theoretical construct is confirmed by data obtained by Eirew et al., who simulated development of tumors from breast cancer patients in xenografts. It was found that the clonal structure of xenografts does not duplicate the clonal structure of the original tumor. Generally, a dominant clone developed from originally polyclonal tumors in xenografts. This clone was underrepresented in the original tumor and, vice versa, originally oligoclonal tumors led to tumors with a high level of heterogeneity in xenografts [45, 46].

CLONAL EVOLUTION OF A TUMOR IN CHEMOTHERAPY

Systematic chemotherapy and target therapy are two of three main methods of tumor treatment. If all tumor cells were equally sensible to therapy, any medical manipulation would lead to destruction of the whole tumor and complete recovery [47], but unfortunately it is not so. When therapy results in significant antitumor effect, it means that before the therapy most of the tumor was represented by cells sensitive to the medication used. Death

of those cells explains the clinical effect — significant (up to full regression) reduction of tumor mass [21, 48]. However, even full regression of clinically detectable focuses does not necessarily mean recovery.

In most cases, due to spatial intratumoral heterogeneity, tumors are only partially sensitive to therapy, and during therapy clonal evolution of the tumor takes place. This may lead to progression (relapse or metastasis) or developing resistance to treatment (Fig. 2). Obviously, relapse and progression after positive therapeutic effect achieved earlier occur due to a resistant clone that was
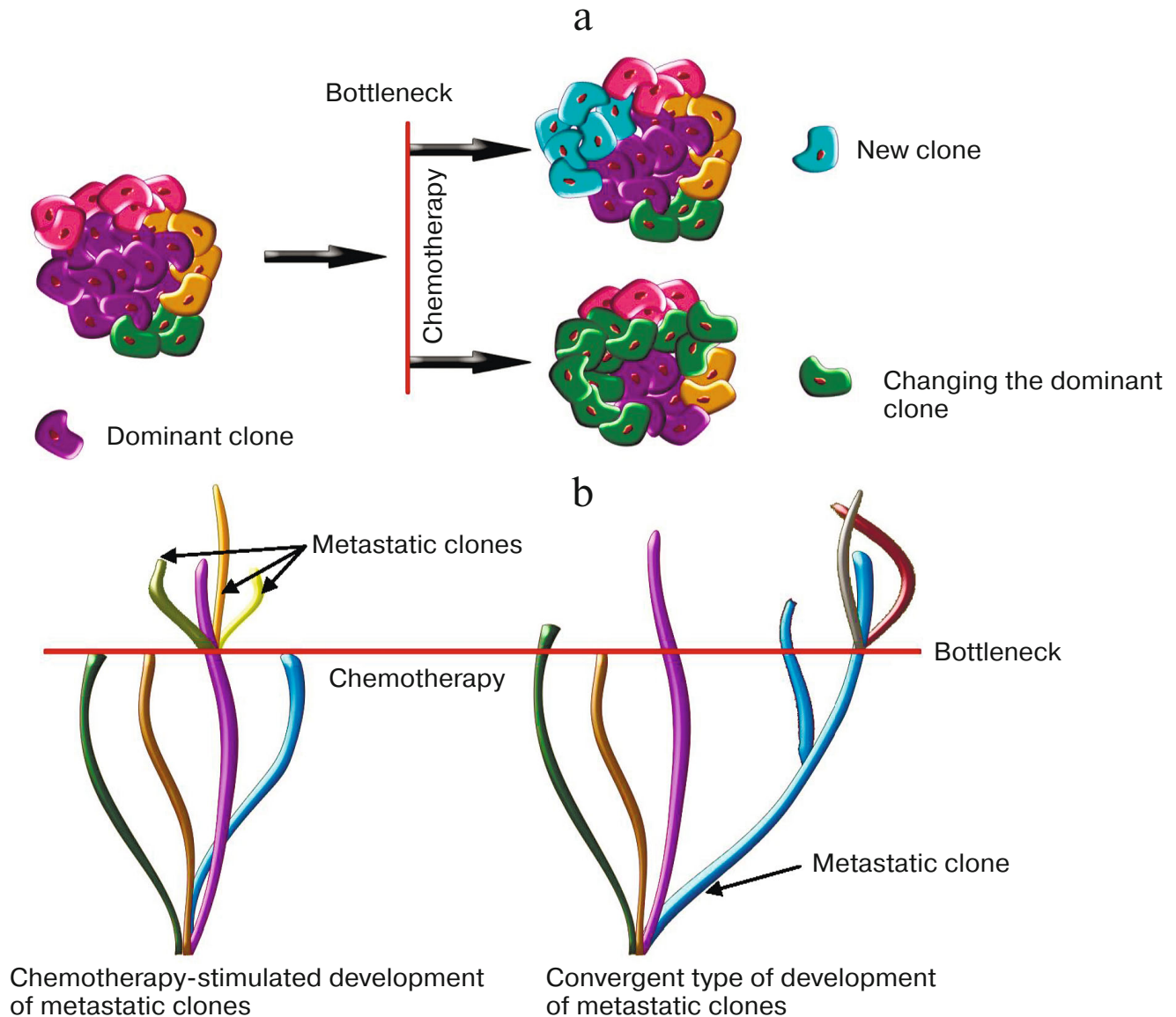

Fig. 2. Chemotherapy-induced clonal evolution of a tumor. a) Two well-known variants of tumor evolution under chemotherapy: development of new clone(s) (blue) due to mutagen effect of chemomedication, and clonal expansion of a minor resistant clone (green) into the space cleared by eliminating the clone that was dominant in the initial tumor, by chemomedication (purple). b) Two types of metastatic clone formation in a tumor. Left-hand side: formation of new clones under chemotherapy. The clones acquire the ability to metastasize. Right-hand side: convergent type of metastatic clone development. Metastatic clones were in the tumor at the early stages of carcinogenesis and evolved along with the initial tumor. Chemotherapy initiated their expansion and formation of clinically manifested metastases.

present before therapy and became dominant after chemotherapy, or it originated during chemotherapy (Fig. 2a). Resistance of this clone is based on features that were likely absent in cells that did not survive therapy (otherwise, they would not die and the clinical effect would not be achieved). After chemotherapy, the population of tumor cells may again be quite heterogeneous, and the clone responsible for resistance to therapy will again be present in the tumor, which will eventually result in tumor resistance. According to the model described by Kreso and Dick, not somatic, but tumor stem cells belonging to different clones are chemoresistant, they survive chemotherapy and initiate clonal diversity of tumor relapse [49].

There are few clinical studies in clonal evolution under chemotherapy. Hematological malignancies are one of the most suitable models in this regard. A study of patients with acute myeloid leukemia performed before and after chemotherapy and thorough analysis of relapses showed that treatment causes cancer cell composition and the array of mutations to change. Comparative study of the genetic landscape in tumor at two stages (before and after chemotherapy) was conducted by Ding et al. [50]. They demonstrated differences in aberrations between primary acute myeloid leukemia and relapses in eight patients. Relapse had two possible variants: in three out of eight cases, the dominant subclones diagnosed in the primary tumor, acquired additional mutations after chemotherapy and developed before relapse. As for the other five cases, a minor subclone developed before relapse, it was present in the primary tumor, and not only did it survive chemotherapy, but it accumulated additional mutations. After chemotherapy, it turned into a dominant relapsing clone.

A full course of chemotherapy of multiple myeloma lead to relapse of a clone that was minor at the time of diagnosis. A thorough analysis describes two primary clones of multiple myeloma, in which the percentage ratio of daughter subclones changed drastically after full-course therapy, i.e. the daughter clone, which was minor at the early stages of myeloma development, not only evolved into a dominant one, but initiated plasma cell leukemia, the most aggressive form of multiple myeloma [51].

Similar data were obtained for another type of tumor − diffuse B-cell lymphoma. Jiang et al. demonstrated two ways of chemotherapy-induced clonal evolution of this oncopathology: the first variant sees a rare clone developing along with the dominant subclone of the tumor; this clone survives chemotherapy, which eliminates the sensitive dominant clone. The minor resistant clone expands into the free space, becomes dominant, and, in the end, initiates tumor relapse. In another case, the minor subclone develops much later during therapy than the dominant one, survives chemotherapy, and initiates relapse [52]. Studies of patients with acute lym-

phoblastic leukemia showed that relapse after chemotherapy includes a host of new mutations; 44% of cases (24/55) involved mutations in the *NRAS*, *KRAS*, and *PTPN11* genes in particular. The authors suggested that chemotherapy plays a key role in development of these mutations and stimulates relapse and resistance [53].

Proof that tumor relapse may occur as a result of evolution of the primary clone during chemotherapy was also presented (using NGS) for chronic lymphocytic leukemia (CLL) [54], glioma [55], and medulloblastoma [56].

CLL patients bearing *TP53* mutations comprise a special group that is hard to treat. *TP53* mutations or 17p deletions diminish response to chemotherapy. The main reason for resistance to treatment is inability of the p53 mutant protein to induce apoptosis appropriately, and dysfunction of this protein is the main reason for genome instability in cells of CLL patients [57]. Using next generation sequencing, the authors analyzed clonal evolution of *TP53* mutations in CLL patients treated (209 patients, median of observations was 61 months) and non-treated (121 patients) at two checkpoints. At the first checkpoint, all 330 patients had the *TP53* gene intact. At the second checkpoint (50-60 months from the first), the group of non-treated patients had only one patient with *TP53* mutations. Whereas 43 of 209 (21%) treated patients had mutation in the *TP53* gene. Overall survival was much lower in the group of patients with mutations of *TP53* in relapse compared to patients with just relapse ($p = 0.03$). The authors noted high risk of mutations during therapy (hazard ratio HR = 0.25, calculated from survival curves (95% CI 0.13-0.47; $p < 0.001$)) and concluded that most *TP53*-mutated clones occur under selective pressure by chemotherapy as opposition to the dominant clone, whereas minor clones are rarely preserved after chemotherapy [54]. This variant of tumor progression simulation by chemotherapy is presented in Fig. 2b (left-hand side). Landau et al. [58] obtained analogous data. Using sequencing, they studied 59 CLL specimens before treatment and of relapses after FC (fludarabine + cyclophosphamide) and FCR (fludarabine + cyclophosphamide and rituximab) regimen chemotherapy. Clonal evolution with development of *de novo* mutations was detected in 97% (57/59) of cases, and in 25% (15/59) of cases *de novo* mutations of the *TP53* were detected. Besides, the authors discovered that new driver mutations are characterized by 1.5-fold higher speed of relapse clone growth in comparison to the primary clone [58].

Anaplastic astrocytoma and glioblastoma are exclusively malignant forms of gliomas; they often relapse after surgery, which is the main reason for mortality from this disease. Johnson et al. suggested a hypothesis stating that genetic changes that stimulate growth of relapses are different from genetic changes in the primary tumor. To prove this hypothesis, they sequenced exomes of 23 primary gliomas and tumor relapses from the same patients.

In 43% of cases, half of the primary tumor mutations were not detected in the relapse, including driver mutations in the *TP53*, *ATRX*, *SMARCA4*, and *BRAF* genes. On this basis, they suggested that tumor relapses are often initiated by tumor clones of the primary tumor at a very early stage of natural evolution, when these drivers were absent. Ten patients were treated with temozolomide, and, during chemotherapy, relapses in six patients acquired new driver mutations in the *RB1* gene and genes (*PTEN*, *AKT2*, *DDIT4*, *ERBB2*, *NF1*, *mTOR*, *PDGRFA*, *PIC3CA*, *TCS1*, *PIC3R1*) of the mTOR pathway, which is inhibited by temozolomide [55]. Similar data were obtained in medulloblastoma, which is the most widespread malignant brain tumor in children. Metastases and the primary tumor had low quantity of common mutations, and the authors suggested that metastases develop from separate rare subclones of the primary heterogeneous tumor, and then new mutations accumulate in the primary tumor along with the metastases due to factors that include chemotherapy [56]. These data are also supported by recent research of American scientists. They studied the primary tumor and relapses after radio- and chemotherapy by temozolomide of 114 patients with glioblastoma. The discovered diversity of new mutations in tumor relapses was vast in comparison to the primary tumor, including driver mutations in such genes as *TP53*, *PTEN*, *EGFR*, *PIK3CA*, *ATRX*, *IDH1*, *PIK3R1*, and *PDGFRA*. They suggested that such high speed of evolution is impossible during treatment and believe that relapsing clones were minor (their frequencies were lower than sensitivity of measurement methods). Besides, in 11% of relapses the *LTBP4* gene was mutated, which leads to *TGFb1* hyperexpression associated with low survival [59]. This situation of tumor progression is illustrated in Fig. 2b (right-hand side).

Occurrence of new clones during treatment was also observed in treating patients with non-small-cell lung cancer (NSCLC) by tyrosine kinase inhibitors — in 50% of cases treatment initiated occurrence of mutation, which prevented antitumor activity of the target medication (due to unavailability of the kinase domain); however, the primary driver mutation in the *EGFR* gene remained [47, 60]. A recent study also showed that on treatment of metastatic NSCLC with activating mutations in the *EGFR* gene (deletion with no frameshift in exon 19 and replacement of L858R in the exon 21) by target medication and inhibitors of *EGFR* (gefitinib and erlotinib), replacement of T790M in exon 20 may occur in the tumor, which significantly lowered the effect of those inhibitors of *EGFR* [61].

The report of Murugaesu et al. presented the results of sequencing of specimens obtained from the patients with adenocarcinomas of the esophagus before and after presurgical (neoadjuvant) chemotherapy, whose main goal is to decrease the volume of the primary tumor (up to complete elimination) to enable surgery in cases of inop-

erable tumors or conservation surgery, and to determine the resistance to postsurgical (adjuvant) chemotherapy. Sequencing was performed for the DNA specimens obtained from different areas of the tumor and biopsy material for each patient. Matched analysis of biopsy before treatment and of operational material of the tumor after platinum-based preoperative chemotherapy was performed for a small number of patients — five people. However, this study illustrates the mutation processes and evolution of this type of cancer after cytotoxic therapy. It was shown that two of five specimens (40%) manifested change in clonal structure in the tumor under the influence of chemotherapy (frequency of C>A mutations in CpC contexts of the tumor DNA increased), which was associated with insufficient response to chemotherapy in these patients [62]. Findlay et al. used whole exome and targeted deep sequencing to study 30 specimens of esophagus adenocarcinoma before treatment and after two courses of preoperative chemotherapy (oxaliplatin–fluorouracil). It was found that sufficient response to chemotherapy is followed by significant decrease in diversity of mutations including driver mutations of such genes as *TP53*, *SMARCA4*, and *ARID1A*, in most cases due to deletion of loci of these genes. The authors also observed significant decrease in clonal diversity of tumors. Vice versa, resistant tumors manifested driver *de novo* mutations in such genes as *TP53*, *SF3B1*, *TAF1*, *CCND2*, *FBXW7*, *SMARC4A*, and *CNTNAP5*, as well as amplification of loci of the *ERBB2*, *CCND2*, *TERT*, and *CCNE1* genes. Besides, patients who did not respond to chemotherapy showed overrepresentation of *TP53* mutations (they were absent in the initial specimen), and this correlated with negative outcome (approximately 20% of patients). Considering the use of deep sequencing, which secures high sensitivity and absence of mutations in the specimen before treatment, the authors suggested that preoperative chemotherapy stimulated their occurrence. And even a short-term course of preoperative chemotherapy may induce rapid evolution of the esophagus tumor genome, and it can acquire resistance to treatment. Good clinical and genetic response is associated with vast SNV variability and high percentage of T:A>A:T mutations in the specimen before treatment, though not with the level of clonal diversity [63].

Change in tumor clonal structure under chemotherapy is also studied in cases of colorectal cancer [12, 41, 64]: the results obtained by Krešo et al. show that KRAS-mutated variants, which secure secondary resistance to monoclonal antibodies against EGFR, are detected much earlier than clinical tumor progression is observed [64]. In other words, development of resistance in one patient may occur due to multiple mutations acquired during treatment [65, 66]. In this context, clonal evolution is at the forefront as the main reason for inefficiency of target medication. In general, clonal evolution of a tumor during chemotherapy and target therapy was described for

many localizations: various hematological malignancies, lung cancer, prostate cancer, esophagus cancer, colorectal cancer, and some others [67].

A study by Litviakov et al. [68] included investigation of clonal evolution of breast cancer during preoperative chemotherapy. The study involved 30 patients (IIA-IIIB), and each woman had her breast tumor CNA (Copy Number Aberration) status studied using the CytoScan HD Array (Affymetrix, USA) microarray before treatment and after FAC (5-fluorouracil, adriamycin, and cyclophosphamide) or CAX (cyclophosphamide, adriamycin, and Xeloda® (peroral form of 5-fluorouracil)) chemotherapy. The CytoScan HD Array microarrays cover 18,500 RefSeq of genes and show, among others,
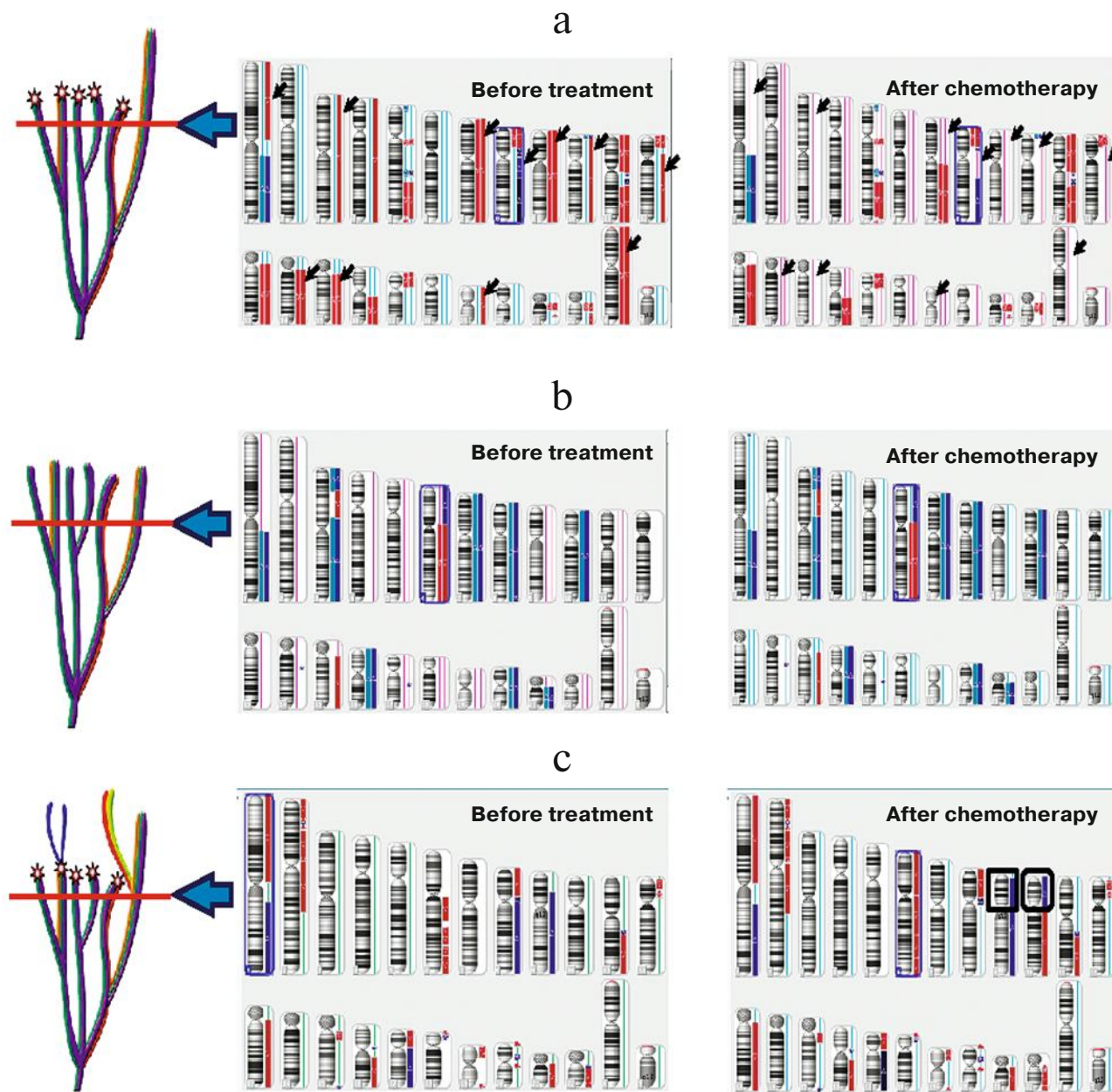

Fig. 3. Three variants of clonal evolution of breast tumor during preoperative chemotherapy (our own data). a) Decrease in number of clones with amplifications and deletions (up to full elimination: four cases of complete morphological and genetic tumor regression) in 13 of 30 (43%) patients (no metastases); b) preoperative chemotherapy did not affect clones with amplifications and deletions in 8 of 30 (27%) patients (no metastases); c) during preoperative chemotherapy, new clones developed in the tumor (in nine of 30 (30%) patients), including clones with amplification in six of 30 (20%) patients, and all six of these patients developed metastases in the period of 1-3 years. The figure outlines three variants of clonal evolution trees of life. Windows of the program for analysis of microarray CytoScan HD Array (Chromosome Analysis Suite 3.0 software; Affymetrix, USA) are illustrated with the image of genetic landscape of tumor before treatment and after preoperative chemotherapy. Blue next to the chromosome represents amplified regions; red represents deleted areas.

mosaic deletions and amplifications of primarily exon-coding sequences of genes with sensitivity of 10% to mutant DNA in contrast with normal DNA. It was shown that change in frequency of CNA during chemotherapy directly correlates with efficiency of preoperative chemotherapy (Spearman correlation coefficient = 0.71, $p = 0.000011$). This may prove to be promising in terms of using the change in CNA frequency to evaluate genetic efficiency of chemotherapy. Another interesting and important result was obtained in terms of the influence of chemotherapy to the clonal structure of a tumor: it was found that during chemotherapy 43% (13/30) of patients had number of mutant tumor clones decrease (up to complete elimination: four cases of complete genetic regression of tumor); in 27% (8/30) cases chemotherapy did not affect the number of mutant clones and frequency of CNA; 30% (9/30) of patients (during preoperative chemotherapy) manifested occurrence of clones bearing new CNA (deletions or amplifications). In addition, six of nine patients manifested development of tumor clones under the influence of preoperative chemotherapy, which brought new amplifications (Fig. 3). In 100% of cases, development of new amplifications was associated with development of hematogenous metastases. In other words, preoperative chemotherapy stimulates metastasis of breast cancer in 20% of cases. All other patients with elimination of clones, absence of effect of chemotherapy on the clones, or patients with occurring deletions did not have hematogenous metastases throughout the five-year period of observation (Kaplan−Meier method, $p = 0.00000$ Log-rank test). This is the first absolute predictive factor when a feature (in this case, it is occurrence of clones with amplification during chemotherapy) is associated with 100% metastasis and absence of the feature is associated with 100% survival [68].

Studies by Chinese researchers showed loss of somatic mutations of the *TP53* and *PIK3CA* genes in breast cancer tissue after preoperative chemotherapy. Tumors from 364 patients before treatment and after chemotherapy were studied using the NGS method. Frequency of somatic mutations in *TP53* or *PIK3CA* were identified in 24.8% of tumor specimens before treatment, but only in 12.1% of specimens after chemotherapy ($p < 0.001$). Patients with *TP53* or *PIK3CA* mutations in tumor before treatment, if they did not become negative in these mutations, demonstrated significantly better complete and relapse-free survival after chemotherapy ($p = 0.008$). The authors suggested that chemotherapy may reduce frequency of mutation in patients with breast cancer, and that it may become a positive predictive factor [69].

Thus, change in clonal structure of a tumor after chemotherapy confirms that chemotherapy can be regarded as driving factor of the tumor evolution process. Some authors believe that survival of minor clones and their expansion during chemotherapy is the main mechanism of development of resistance and progression after

chemotherapy. Other researchers suggest that the speed of tumor evolution during chemotherapy drastically increases and the main mechanism of resistance and progression is development of new genetic anomalies and driver mutations in tumor cells. There is even an opinion that states that chemotherapy may stimulate malignant transformation of tissue that was normal before treatment. In any case, this states the necessity of thorough study of processes of clonal evolution of a tumor during chemotherapy to prevent stimulation of tumor progression and to search for new markers for personalized treatment. This line of research is also urgent in terms of detection of mechanisms of development of resistance and for creation of new predictive markers [70].

APPROACHES TO TUMOR TREATMENT BASED ON CLONAL EVOLUTION

Currently, researchers have already started developing approaches to treatment based on clonal evolution of tumors. Without a doubt, precision therapy should be regarded as an achievement as it is based on determination of stages of natural evolution of a tumor in every patient [71]. While studying multiple parts of a tumor in one patient separately using NGS, the tree of life of natural clonal evolution is modeled. The primary driver common for all parts of a tumor is determined, and target medication is set for it. Unfortunately, this approach can only be applied to tumors of monoclonal origin (Fig. 1a) whose natural evolution can be illustrated as a "tree of life". Applying this approach to tumors of polyclonal origin (Fig. 1b) or any type of natural clonal evolution different from the "tree of life" will encounter significant difficulties.

Clonal evolution is one of the main reasons for target and chemomedication inefficiency. In terms of predicting the efficiency of medication therapy, it is very important to study the speed of clonal evolution during treatment: to destroy a tumor, the speed of tumor cell elimination must be significantly higher than the speed of clonal evolution of the tumor.

Without a doubt, we can state that spread of metastases is strongly connected with clonal evolution of a tumor, including clonal evolution during treatment [72]. In this regard, the ability to predict the reaction of a tumor to treatment and metastasis during treatment is of great clinical interest. It is necessary to consider the opinion of certain researchers on very high speed of chemotherapy-induced evolution of tumors, which leads to development of new resistant and relapsing clones [58, 63]. It is impossible to ignore the increasingly widespread data indicating that chemotherapy stimulates metastasis or relapsing in almost 20% of patients [54, 63, 68]. All this confirms the need to question almost all modern instructions for chemotherapy now and to start working on

developing new instructions that take into consideration the pace of chemotherapy-induced evolution and the probability of development of resistant and progressing clones. Prescription of chemotherapy must be personalized and predict the probability of metastasis stimulation in a patient.

The next approach, which may already be developing, is formulation of methods of genetic evaluation of chemotherapy efficiency, as significant progress has been made in this line of research [59, 62, 63, 68, 69]. It is likely that in the near future genetic evaluation will replace morphological study of tumor and study of residual tumor to determine drug pathomorphism in particular. Genetic evaluation will help determine clonal changes in residual tumor, personalize prescription of postoperative target or chemotherapy, and predict the outcome of the disease. Probably in the foreseeable future, multi-stage antitumor therapy, based on knowledge of mutations acquired and eliminated in clonal evolution during treatment, will enhance the rates of survival and substantially increase longevity in various cases of malignant neoplasms.

Another idea, which was first put forth at the Nineteenth Russian Oncologic Congress in 2015, is to single out the markers of metastatic clones and to determine their presence in an operable tumor before systemic treatment. If metastatic clones are present in the tumor, systemic therapy may be prescribed with the goal of eliminating these clones. Because the clones are already present in the tumor, the risk of stimulating metastasis is low, but there is a probability of elimination of metastatic clones and of increase in survival of patients. At the same time, if there are no metastatic clones in the tumor, systemic chemotherapy is not mandatory and treatment may go to surgery. The Research Institute of Oncology of Tomsk National Research Medical Center has begun clinical trials of such an approach to prescribing preoperative chemotherapy for patients with breast cancer.

Development of approaches to manipulating clonal evolution of tumors may become a new direction in treatment of malignant tumors. It is already clear that homogeneous tumors respond to treatment better in comparison to tumors with high-level clonal diversity. Methods of creating the conditions for formation of homogeneous tumor may be developed, for example, it is possible to form the dominant clone artificially, which would replace all the other minor clones. The idea is that the tumor should be able to fight itself, and for this, it is necessary to stimulate competition between clones in the tumor as much as possible. Methods of genetic editing may be applied to impose a driver mutation for which there is a target medication. Then the tumor is put into conditions of stabilizing or metronomic chemotherapy (each day the patient takes a small dose of chemomedication, which is different from the standard chemotherapy, when every three weeks the patient takes maximum dosage) so that the clone with the artificial driver is not sensitive to it, and

this will give it the opportunity to dominate and to replace minor clones. In some time, at the last stage, target therapy is used directly onto the artificial driver, which destroys the dominant clone.

Thus, study of mechanisms of natural and chemotherapy-induced clonal evolution of various tumors will enable determining the order of molecular and genetic events that take place in development of a form of cancer, enable connecting molecular and genetic changes with the clinical course of the disease, metastatic potential, and response to the therapy, which eventually will enable personalized treatment of each patient and manipulation of clonal evolution of tumors.

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