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POINT OF VIEW

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IN SEARCH OF MOLECULAR APPROACHES TO IMPROVING CANCER THERAPY EFFICACY

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The study of genome rearrangement sites using full genome sequences is an important approach to revealing the nature of cancer and finding effective ways for cancer treatment. The progress in DNA sequencing could make the procedure of whole genome reading close to routine procedure of lower cost. The personal analysis of rearranged ends (PARE) method used for rearrangement study is reviewed. PARE allows identifying of individual cancer biomarkers making personal medicine possible. Also, the progress in "liquid biopsy" technology based on detection of circulating tumor cells in the patient's blood is shortly summarized. Another important approach is the discovered phenomenon of synthetic lethality causing cancer cell death due to appropriate combination of mutations in different genes or inhibitors of their protein products. Studies of genome rearrangements and synthetic lethality are considered promising for the development of effective cancer treatment approaches.

Key Words: **PARE, circulating tumor DNA, circulating tumor cells, liquid biopsy, synthetic lethality, cancer cell.**

Obviously, there comes a time in oncology speaking in biblical language to "gather stones" and to evaluate the significance of molecular diversity detected in tumor cells. Such a feeling may emerge due to the organization of the first systemic discussion of numerous results obtained in individual cancer treatment. Such discussion was organized by the Ist International Congress on personalized treatment of cancer (Controversies in Personalized Oncology Treatment) that took place in Barcelona on 7–10 March, 2013 [1]. Prospects of personalized medicine are mostly based on advances in the study of the individual characteristics of tumor DNA sequences. DNA sequences rearrangements especially frequent in cancer genome could currently be localized allowing the construction of individual patient's oncomaps. Another important approach is the discovered possibility of the so-called synthetic lethality. This term is related to tumor cell killing with the use of individual molecular combinations targeting activity of special enzymes or induction of mutations. These novel approaches can be somewhat simplified using the so-called "liquid biopsy" as the recently discovered possibility of manipulating with cancer patient DNA and separate circulating tumor cells (CTC) present in blood and possibly in other fluids of the organism. Here we discuss some of the molecular approaches that could improve treatment of cancer patients

The study of the individual characteristics of DNA in cancer patients is becoming easier due to new technologies: comparable genome hybridization (CGH) and DNA sequencing called New Generation Sequencing (NGS). Possibility of full genome sequencing allows detecting differences in genetic texts of cancer patients in comparison with the genome of healthy individuals. Such a healthy genome for the comparison purposes is called reference genome or reference assembly using the DNA sequencing data from a number of healthy donors. Useful information can currently be obtained by comparison of a) nucleotide sequences of healthy and cancer genomes of different individuals, b) genomes of normal and malignant cells of the same organism, and c) nucleotide sequences of tumors of different histological types. Thus, the developed technologies opened a powerful way for the genetic mapping [2]. Analysis of oncomaps allowed separate the genes carrying cancer-related mutations into two groups: driver and passenger as to whether they do or do not influence the malignant cell reproduction rate and hence the growth of the tumor [3]. Interesting, could the genomic rearrangements present in cancer patient be divided alike?

The opened possibilities to study sequence alterations in full cancer genomes draw attention to genome rearrangements. Until recently the main efforts in tumor DNA studies were focused mainly on two things. The first is single nucleotide polymorphism (SNP) presented by different point mutations and the so-called indel mutations (associated with insert or loss of nucleotides-deletion). And the second one is mainly due to the processes of nucleotide loss or conversely amplification that change the number of gene copies in genome. Previously it was called CNV — copy number variation, and later a new term aCGH (array comparative genome hybridization) appeared. Numerous sequence alterations found in cancer patient DNA are currently covered by specially developed databases. As an example 954,247 mutations in 2680 exomes of 14 cancer types could be studied [4]. These databases include The Cancer Genome Atlas — TCGA data; COSMIC — Cancer Gene Census; CRAVAT — Cancer-Related Analysis of VArients Toolkit, and others that are comprehensively summarized in the work [4 and references therein].

In recent years the genetic rearrangements in cancer cell genomes and their functional role are of particular interest in connection with the previously established fact of aneuploidy in tumor cells [5–9]. Aneuploidy

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Abbreviations used: aCGH – array comparative genome hybridization; CGH – comparable genome hybridization; CNV – copy number variation; CTC – circulating tumor cells; DSB – double strand brake; DTC – disseminating tumor cells; LCM – laser capture microdissection; NGS – New Generation Sequencing; PARE – personal analysis of rearranged ends; SNP – single nucleotide polymorphism; SSB – single strand brake.

is characteristic to cancer cell as well as the inherent instability of cell cancer genome leading to numerous genomic rearrangements and chromosomal chaos [5–7]. Many authors have addressed a question how aneuploidy and genome instability may be associated [3, 10]. In the study [10] there have been compared the results of cancer cells study at the level of karyotypic changes and that of individual genes mutability, without going into details on the genes being drivers or passengers. The most informative name of a novel approach is the classic one "Whole genome vs selected gene panel approach" [11].

The possibility of full genome sequencing and detection of genomic rearrangement sites allows experimental studies of deregulation of cancer cell work as a functional single system. Study of genome rearrangements may deepen insights on cell organization.

Study of genomic rearrangements using PARE method. Development of high-tech methods of sequencing, extremely optimized and less expensive for reading full genome texts, even very large and complex, provided the possibility of detecting various reorganizations of the genome, in particular, changes in nucleotide sequences resulted from formation of different gap(s) and illegitimate reunion of different genome fragments.

An efficacious method to study the genomic rearrangements in cancer cells is a method called PARE (personalized analysis of rearranged ends) [12]. We briefly describe here this technology.

It is clear that full genome sequences could be compared in the manner base by base. However, because the patient genome size is about 3 billion nucleotides, the work would be too expensive and unjustified for clinical use. At the same time, simultaneous examination of only short sequences at both ends of the same DNA molecule of fragmented genome is the way to simplification. The known distance apart both DNA molecule ends allows getting rid of the nucleotide content between the two ends. The identification of these end sequences occurs as if in paired form, in the form of these ends mating (mate-paired ends). Then mapping of these ends to fully sequenced genome significantly simplifies and reduces the cost of finding rearranged sites in genome. Using this approach, it is possible to consider the influence of repeated and amplified sequence first of all when comparing molecules belonging respectively to the genomes of normal and malignant cells. So, this technique accounting nucleotide changes at sites of repeated and amplified sequences, allows identifying such a combination of nucleotide text in the molecules of cancer genome that is not detected in the molecules of a fragmented genome of normal cells. Detection of molecules that carry such a nucleotide combination indicates that these molecules come from genome sites where rearrangement via both intra- and interchromosomal translocations occurs.

To characterize the logic of an approach to study genetic rearrangements using PARE we briefly describe one example. Genomic DNA is fragmented using mechanical efforts and ends of molecules obtained are repaired using biotin label. Then such molecules are circulated and cleared of noncircular DNA using nuclease digestion. Circular molecules obtained are fragmented again, and catching biotin label using magnetic beads with the streptavidin allows obtaining of fragments of uniform size that contain only the end sequence of the same original molecule. The ends of the DNA molecules obtained in such a manner are also repaired and are surrounded by the sequences of special adaptors and primers that allow sequencing 25 nucleotide end tags. Performing emulsion PCR allows landing on a magnetic bead of only one DNA molecule. In this manner the library of end sequences can be obtained and fixed on a hard surface for sequencing in the form of amplicons, or DNA colonies, or using new terminology DNA clusters. In the resulting mate-paired end library, different molecules of the genome are sequenced. Modern equipment and methods allow simultaneous sequencing up to 500 000 of such molecules (massive parallel sequencing) per a sequencer run. Currently, nucleotide sequencing exploits two principles: sequencing by using template synthesis, classical Sanger sequencing and sequencing by oligonucleotide ligation [13]. There has been great progress in the development of equipment, analyzing signals produced by both types of sequencing.

The success of the molecular approach to finding individual tumor markers based on the PARE method and its practical use in medicine has been demonstrated in the work by V. Velculescu et al. [12]. In the referred work the pair ends study approach originally designed by Applied Biosystems SOLiD system was used. Using the reference genome, studying the genome of solid cancers compared respectively to the patient healthy tissue genome, the authors analyzed the short end sequence for each DNA sample of roughly 40 million reads. The positions of these reads at the reference genome were defined exactly. The total nucleotide number of analyzed end sequences was increased by the value of nucleotides between the ends of the studied molecules. Considering the study of the number of mate-paired end tags molecules represented in the library, the performed sequencing resulted in 18-fold physical coverage of the human genome, i.e. with very high probability this analysis covered the whole genome completely.

The detection of individual cancer biomarkers. An important result of the work [12] was the demonstration of the discovery of an individual oncomarker in a colon cancer patient and the detection in the blood plasma (possibly in other body fluids) of tumor mutant DNA with modifications that distinguish it from normal cell DNA. The possibility of manipulations with the cancer cell DNA circulating in the patient's blood was convincingly demonstrated.

It turned out that the mutant DNA, identified only in the tumor cells of a studied patient is characterized by a translocation of sequences between 4 and 8 chromosomes — 4: 8 translocation. Two methods were used to find it. According to one of them molecules that contained sequences belonging to different chromosomes have been selected from the end tag libraries and the genome text was studied at the distance between end tags equal to 1 kb. If an unusual combination of nucleotides has been detected in this group of molecules at least 5 times, such

carriers have been chosen to further research using PCR primer specific for translocation breakpoints. In the case of healthy DNA corresponding PCR products were not found, by they were identified in case of the cancer DNA.

The second method for search of rearranged sequences in cancer cells using mate-paired end sequencing approach allows distinguishing between real translocation breakpoints from those that appear due to genome repetitive and amplified sequences. In this case, the work with tags was carried out on the 3 kb genome sequences. Determination of tags density occurrence on a single molecule and on the total number of molecules of this group allows determining which sites found are related to genome amplification processes and which are real rearranged sequences of a cancer cell. Studying in this manner DNA from 5 different cancer patients, the translocation 4: 8 was identified only in one of them. The particular value of this result is that no comparison of the cancer and normal cell genomes is needed. This halves the cost of the analysis making the price acceptable to the broader range of patients.

Identification of 4: 8 marker in a cancer cell genome has enabled to determine the sensitivity of the measurement in the presence of various DNA impurities originated from healthy tissues. It turned out that using a primer annealing to 4: 8 rearrangement breakpoint allows revealing a cancer cell genome equivalent in the presence of 390 000 normal genome equivalents. So, in the plasma of a patient blood the presence of tumor DNA can be detected at the concentration less than 0.001% of the content of all DNA in the sample. The capability to detect the circulation of the mutant DNA in the body simplifies, accelerates and diversifies the approaches to various cancer research. It is clear that this identified rearrangement of sequences could be exploited not only as a personal biomarker but also allows studying biochemical and physiological changes induced in the cell due to such rearrangement.

Moreover, an individual 4: 8 chromosome translocation biomarker simplifies the study of the efficacy of chemotherapy because it improves monitoring of the marker in blood samples. It opens the prospect for an individual cancer therapy. However, this approach may face certain limitations due to malignant cells heterogeneity observed in the same tumor that is associated with the cancer genome instability. The tumor cells at any given moment are presented in the form of different clones, as well as their different subclones. In the work [14] it has been shown that the study of genomic polymorphism of fully sequenced tumor DNA exons allows comparing the level of clonal heterogeneity and their proliferation rates.

We note here also that the heterogeneity of tumor cells stimulates developing the technology that allows the selection of a single cell; an accurate characterization of DNA from such a cell improves characterization of the studied clone and its different subclones. Obtaining of such cells is possible using with the aid of a laser technique, so-called Laser Capture Microdissection — LCM [15]. The DNA obtained in this way contains sequences of a full genome. By means of amplification and fragmentation it could be used for preparing a library of molecules ready for the mate-paired ends sequencing and identification of rearrangement sites.

Circulating tumor cells. The study of CTC in peripheral blood of patients with solid tumors of epithelial origin is also currently an area of intense studies [16]. Moreover, it was found that tumor progression is accompanied by appearance of disseminating tumor cells (DTC) homing mainly in the bone marrow where they can stay dormant for years due to yet undisclosed reasons [17–19].

Various sensitive methods of isolation of CTC from blood including the "liquid biopsy" are developed. Methods are based on different principles: the use of differences in physical properties (cell density, their size), differences in gene expression profile (the cells express the marker proteins like adhesion molecule of epithelial cells — EpCAM and proteins of cytoskeleton filaments — cytokeratin). The status of these markers can be evaluated by immunostaining, proper results of RT-PCR reactions and special EPISPOT analysis [20].

Synthetic lethality. The distinctive feature of cancer cell is its genome instability and emerging repair defects that make highly probable the mutagenesis processes. High levels of mutagenesis in tumor cell can decrease the efficacy of targeted therapy. Therefore, the success of chemotherapy depends on the ability to inhibit mutagenesis processes.

The studies of different gene mutation effects showed that when mutations in some genes are induced in cells separately there are no large biological effects, except minor phenotypic changes. However, the simultaneous mutations in few different genes may result in cancer cell death that is called synthetic lethality.

A phenomenon called metabolic bypass is related to cell survival in the conditions of the loss of a gene leading to total absence of an enzyme activity [21]. It means an existence of some biochemical compensating mechanisms buffering the possible effects of emerging genetic changes. A special review [22] is dedicated to the principles for the work of such buffer mechanisms. Moreover, in a chapter of the book [21] we have shown that an efficacious approach to the study of the cellular potential buffer mechanisms might be the study of artifacts revealed during gene cloning using genetic complementation methods. Interestingly, these artifacts may belong quite to different metabolic pathways.

So, if the synthetic lethality can be an efficient method for cancer cells elimination from the body, the search of such possible combinations is perspective. Here we present some results of studies in the field of synthetic lethality.

Simultaneously the two groups of researchers have found that treatment of breast cancer cell cultures with a target inhibitor of poly-ADP-ribose polymerase (PARP) resulted in massive cell death [23]. PARP is involved in the reparation processes, in particular, in repair of single strand breaks — SSB using base excision repair BER. When PARP "senses" SSB it undergoes structural change, interacts with coenzyme nicotinamide adenin dinucleotide (NAD^+) and cleaves it producing nicotinamide and ADP-ribose that PARP polymerises to highly negative

chains — PAR. As a result of this reaction, a reparative active protein complex containing PAR-PARP is formed at the site of DNA reparation. Interestigly, when PARP binds its inhibitor, the enzyme interacts with DNA, but can't dissociate from it what makes DNA replication impossible [24]. It was studied why breast cancer cells unlike healthy cells die when they were treated using the inhibitor that competes with NAD^+ for binding with the PARP and thus disabling the enzyme of capacity to repair DNA strands breaks. It turned out that genome reparation is carried out through various mechanisms, and some of them are overlapping. It is known that in breast cancer patients there is malfunction of BRCA 1 and BRCA2 genes. The first gene is involved in repair of double strand breaks — DSB and the second one is responsible for homologous recombination. However, if these genes do not function in breast cancer cells because of mutations induced then PARP begins to fulfill their work. Clearly, the PARP inhibitor deprives the cell of the last chance to maintain its genome intact and emerged in this case synthetic lethality dooms cell to death. Currently the role of synthetic lethality potential in conjunction with that of the reparation processes for the successful treatment of breast cancer is intensely studied [25–27].

The repair processes and synthetic lethality in the case of solid tumors is thought to be influenced by tumor microenvironment. Using model cell culture experiments it has been shown that in condition of hypoxia the genes coding for proteins involved in homologous recombination are not expressed and no DSB reparation occurs. If PARP inhibitor is used, the massive cell death is observed [28].

So, the original technology of possible reading the large arrays of DNA sequences known as New Generation Sequencing allows working with the full genome sequences reliably finding the places of genomic rearrangements. Study of genome reorganizations raises many questions for fundamental research. Currently, new opportunities, namely, the study of circulating tumor DNA and CTC in the manner of "liquid biopsy" have appeared. High levels of mutagenesis observed in cancer cell due to many intrinsic factors (for example induction of APOBEC mutagenase, imbalance in synthesis of DNA precursors) speaking philosophically represent both evil and good. The first is frequent loss of cancer cell sensitivity to target drugs. The second is the possibility of creating lethal combination of mutations leading to possible synthetic lethality of the cell. The important task is learning how to do this probability useful and effective for clinical purposes.

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