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PhD THESIS

NEXT GENERATION SEQUENCING (NGS) AS A METHOD FOR NEOEPITOPES IDENTIFICATION IN SOLID TUMORS

ABSTRACT

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LIST OF PUBLICATIONS

1. **Harich OO**, Olteanu GE, Mihai IM, Benta M, Gavriliuc OI, Paunescu V, Bojin MF. Unique Growth Pattern Presentation of a Papillary Renal Cell Carcinoma. *Diagnostics* 2022; 12: 1904. (Impact factor = 3.6) https://doi.org/10.3390/diagnostics12081904
2. **Harich OO**, Anghel S, Tatu C, Tanasie G, Paunescu V, Panaitescu C. Precision diagnostic methods in solid tumors - next generation sequencing (NGS) *Fiziologia-Physiology*, 2023; 1(104): 47-52.
3. Koteles MM, Vigdorovits A, Kumar D, Mihai IM, Jurescu A, Gheju A, Bucur A, **Harich OO**, Olteanu GE. The Intersection of AI and Pathology: Analyzing the Grading of Ductal Carcinoma of the Breast through Deep Learning and Traditional Assessment. *Diagnostics*, 2023; 13: 2326. (Impact factor = 3.6) https://doi.org/10.3390/diagnostics13142326
4. **Harich OO**, Gavriliuc OI, Ordodi VL, Tirziu A, Paunescu V, Panaitescu C, Bojin MF. In Vitro Study of the Multimodal Effect of Na+/K+ ATPase Blocker Ouabain on the Tumor Microenvironment and Malignant Cells. *Biomedicines*, 2023; 11: 2205. (Impact factor = 4.7) https://doi.org/10.3390/biomedicines11082205

INTRODUCTION

Cancer is a major health problem worldwide, with 19 million newly diagnosed patients in 2020, the most common types being breast, lung, colorectal, prostate, gastric, liver and cervical cancers. The mortality rate in 2020 was over 10 million, the main causes of death being lung, colorectal, liver, gastric, breast, esophagus and pancreas tumors.

The situation is similar in Europe, and in Romania there were over 100,000 new cases registered in 2020; breast, colorectal, lung, prostate and bladder tumors account for 50% of cases.

In the context of the continuous growth of tumor pathology, new methods of screening, diagnosis and treatment are sought, which lead to the improvement of methods of early identification and treatment specific to each type of tumor.

New generation sequencing (NGS) has had a significant impact in the field of solid tumors, bringing multiple benefits in the understanding and management of these conditions, such as: identification of driver mutations, comprehensive genomic profile, monitoring of tumor evolution, identification of therapeutic options, prognosis and risk

management. Next-generation sequencing (NGS) plays a critical role in understanding and addressing solid tumors.

New generation gene sequencing (NGS) is a method widely used today, both in tumor screening (gene sequencing from liquid biological samples – liquid biopsy) and in the precision diagnosis of tumors that are constituted and diagnosed by conventional means. This method also allows the indication of a targeted therapy, both from the arsenal of classical chemotherapy and from the new specific immunotherapies. Programs for the analysis and interpretation of sequencing data, which, through access to large public and private databases, enable the identification and access of cancer patients to clinical trials that address mutations identified by said methods.

In this context, the present study fits the current research trends in cancer, proposing a panel of gene investigations by NGS, followed by the identification of new therapeutic targets in solid tumors.

RESEARCH OBJECTIVES

The present work had 3 major research objectives:

1. Performing gene sequencing for patients with solid tumors of different morphopathology types, through new generation techniques (NGS) and identifying gene mutations associated with each type of tumor;
2. Determination of somatic gene mutations, common to all types of solid tumors investigated according to allelic frequency and location, as well as identification of induced changes in the expression of proteins associated with each gene;
3. Identification of neoepitopes presented by tumor cells in the context of molecules from the major cytocompatibility system (MHC) class I, as potential anti-tumor therapeutic targets or for identification by cells of the immune system (cytotoxic T lymphocytes) and triggering of intrinsic anti-tumor response.

GENERAL PART – LITERATURE REVIEW

Cancer is a major health problem worldwide, and the number of patients is increasing rapidly. The latest statistics show that more than 19 million new cases were diagnosed in 2020, with the highest figures recorded for breast, lung, colorectal, prostate, gastric, liver and cervical cancers. In 2020, nearly 10 million people died of cancer, with lung, colorectal, liver, gastric, breast, esophageal, and pancreatic tumors being the leading causes of death.

The situation is similar in Europe (4,497,329 new cases), as well as in Romania with approximately 100,000 new cases registered in 2020; breast, colorectal, lung, prostate and bladder tumors account for 50% of cases.

Genetic mutations are a change in the sequence of nucleotides on DNA that is not a direct result of genetic recombination. Mutations can be differentiated into induced

mutations or spontaneous mutations. Induced mutations are the result of a mutagen acting on DNA, while a spontaneous genetic mutation is the consequence of a mistake that can occur naturally in various endogenous processes. An example of these endogenous processes is the replication of DNA itself. A mutation can be either an insertion/deletion or a point mutation, the latter also known as a base pair mutation or base pair substitution.

Oncogenes are responsible for encoding various proteins that have a major role in the control and regulation of apoptosis and cell proliferation. A mutation within the gene can lead to the activation of an oncogene, either by positioning it next to an enhancer element or by amplifying it. Not only does a mutation have the potential to initiate such a process, but genetic translocation can also lead to this process. The best-known example of an oncogene produced by chromosomal translocation is the formation of the Philadelphia chromosome, t(9;22)(q34;q11), often leading to the development of hematopoietic tumors.

Next-generation sequencing (NGS), also called massive parallel sequencing, is characterized by a very high throughput compared to Sanger sequencing. This technique simultaneously sequences multiple DNA fragments from a previously prepared library, thus archiving a much higher sequencing rate than first-generation sequencing, which allows only one molecule to be sequenced at a time. NGS usually involves three steps: first, fragmentation and library preparation, second, clonal amplification, and third, the actual sequencing process followed by reconstruction of the whole genome sequence.

The DNA sequencing method using a semiconductor ion sequencer is based on the detection of hydrogen ions that are released during DNA polymerization. Addition of a dNTP to a DNA polymer results in the release of a pyrophosphate and a hydrogen ion, thereby changing the pH, which can then be measured by an ion-sensitive layer on the semiconductor chip. During the sequencing process, the chip is alternately flooded with one of the four DNA nucleotides, each time a nucleotide is incorporated, a hydrogen ion is released. The change in pH is then measured and converted to voltage. The change in voltage indicates that a nucleotide is incorporated and therefore a base has been determined. This is repeated every 15 seconds with a different wash of nucleotide across the chip. The Ion Torrent technology does not use fluorescently labeled dNTPs, but rather unmodified bases, as it is sensitive to changing hydrogen ion concentration. The release of hydrogen ions is directly proportional to the number of incorporated dNTPs, therefore, in a base repeat (e.g. TTT) more than one nucleotide is added simultaneously, leading to a larger change in pH. The specialized semiconductor chips allow the simultaneous detection of millions of such changes, quickly determining large amounts of sequences. In a 2.5-hour runtime, the Ion Torrent is capable of processing up to 10 Gb of sequencing data.

NGS assays designed to explore mutational status in cancer-associated genes beyond specific hotspot mutations have not been characterized. Knowledge of these data is particularly important in the choice of treatment for patients with rare types of cancer, including pediatric patients. Genomic data are valuable when the identified genes are related to clinical data, the type, number, responses and duration of previous treatments

and other clinicopathological characteristics. As such, informed consent must be obtained from all patients before their anonymized data are used by researchers and clinicians.

The scope of clinical utility of NGS-based cancer tests is beginning to expand substantially. The information provided by NGS has become valuable in therapeutic decisions, as we now better understand both the co-occurrence and mutual exclusion of mutations in cancer-associated genes. For example, the presence and number of mutations are associated with a response to checkpoint-type immune inhibition, and an increased number of mutations may have therapeutic implications.

It has been established that cancer vaccines that trigger T-cell activation against tumor antigens can be beneficial for cancer patients, although many improvements in vaccination strategies are still needed to achieve long-term patient survival. Various immunogens were tested, ranging from minimal CTL epitope to full-length recombinant protein. The minimal CTL epitope, typically 9–10 amino acids in length, induced a limited number of effector T cells, associated with reduced clinical efficacy, likely due to CTL anergy. This anergy likely resulted from exogenous loading of the short epitope and direct presentation to CD8 T cells, thereby bypassing intracellular antigen processing by DCs and co-signaling by mature DCs. On the other hand, vaccination with full-length recombinant proteins is not the best alternative. In vivo studies in mice showed that all intracellular cross-presentation pathways were more efficient when synthetic long peptides (SLPs) were used than with the full-length antigen. Therefore, SLPs, usually defined as 25–35 amino acid (AA) long peptides comprising a well-defined CD8 epitope expanded to include putative CD4 epitopes, are currently considered the most effective immunogens. These are usually administered as a mixture of 10-12 such constructs, to cover a wide range of HLA haplotypes and/or a wide range of epitopes. Long synthetic peptides have demonstrated clinical efficacy against HPV-induced cervical and vulvar neoplasia and have recently been used in melanoma patients to vaccinate them against tumor neoantigens. In most reported cases, the choice of SLP was primarily based on a defined CD8 epitope and assumed the presence of an auxiliary CD4 epitope in the vicinity. An alternative strategy for SLP vaccine design relies on careful selection of well-defined CD8 and CD4 epitopes for which there is a broad repertoire and/or elicit strong immune responses. Selection of both CD4 and CD8 epitopes offers a wide range of opportunities: separating naturally overlapping epitopes, binding epitopes that are far apart on the natural antigen, or creating chimeric epitopes containing a CD4 epitope from a antigen coupled to a CD8 epitope from another tumor antigen. Thus, universal CD4 helper epitopes have been described, capable of binding to a wide range of HLA haplotypes and thus eliciting responses in a large patient population.

In this context, two basic clinical trial designs for testing genomically targeted cancer therapies have been developed:

1. *Basket trials* place all patients with tumors expressing the same genomic target into the same category (basket) allowing patients to receive appropriate, targeted therapy.

2. *Umbrella trials* involve investigating several targeted therapies and enrolling specific groups of patients in different studies depending on their tumor genotype. In both types of studies, a state-of-the-art sequencing assay that allows for the detection of multiple alterations can provide information that allows patients to be included in one of the available study cohorts (Figure 1).

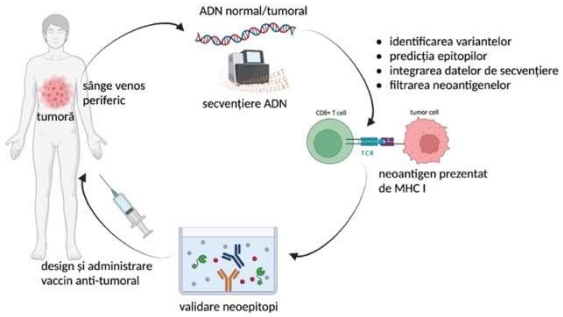

Fig. 1. Graphical abstract of research phases

SPECIAL PART – PERSONAL CONTRIBUTIONS

1. MATERIALS AND METHODS

1.1. Subjects included in the study

The subjects included in the study were oncology patients, with an established diagnosis, who voluntarily presented themselves at the "Pius Brînzeu" County Emergency Clinical Hospital, Timișoara, the Center for Gene and Cell Therapies in Cancer Treatment - OncoGen with the biological samples taken previously in specialty departments in the course of oncological diagnosis and treatment; the biological samples included in the study were paraffin blocks with tumor/normal tissue, which were previously used for diagnosis, staging and establishment of conventional anti-tumor therapeutic strategy. In the framework of the doctoral study, the results obtained through additional investigations (New Generation Gene Sequencing - NGS) were used exclusively for research purposes.

Inclusion criteria of subjects in the study: patients with diagnosed neoplasic pathology - solid tumors, who voluntarily offered the biological material (paraffin embedded tumor tissue), exclusively for research purposes; Caucasian male and female subjects from both urban and rural backgrounds, aged 18 to 80 years; with capacity to understand the information presented to the subjects and willingness to sign the informed consent.

Exclusion criteria: vulnerable subjects/populations.

The study conducted at Emergency County Clinical Hospital "Pius Bînzeu" Timișoara is a prospective cohort study, basket-type, which involved the analysis of paraffin embedded biological samples obtained from patients who were previously diagnosed with oncological pathology - solid tumors, in order to identify the gene mutations present in the tumor tissue. The results of gene sequencing analyzes were used exclusively for

research purposes, to identify and establish the frequency of SNV-type gene mutations from a panel of genes involved in tumor development.

The prospective basket-type cohort study was conducted for 3 years, between 2020-2023, on a group of 100 cancer patients (solid tumors), being divided according to the site of the primary tumor into: breast cancer (n=33), genitourinary tumors (n=16), lung cancer (n=15), digestive tract tumors (n=15), other types of solid tumors (n=21). The category of genitourinary tumors included ovarian tumors, adenocarcinomas of the prostate, kidney and bladder tumors. Liver tumors, colon cancer and gastric cancers were included in the category of digestive tract tumors. In the category of other types of solid tumors, tumors with a low incidence and common ectodermal origin of the tumor tissue, such as melanomas and brain tumors - glioblastomas, were included.

1.2. Methods for next generation sequencing (NGS)

Samples: paraffin-embedded tumor tissue

The procedures used in this study were performed on paraffin blocks containing tumor tissue, which were processed for nucleic acid (DNA) purification and next generation gene sequencing (NGS). The results obtained were used exclusively for research purposes, patients did not benefit and were not exposed to risks.

DNA extraction kits: QIAamp DNA Blood Minikit, Pure Link Genomic DNA kits for DNA purification, QIAamp DSP DNA FFPE Tissue Kit; DNA measurement kit: Qubit dsDNA HS Assay; Analyzed genes panel: Hotspot Panel – 49 genes and 207 hotspots; Analysis device: Personal Genome Machine (PGM) Ion Torrent, Ion Gene Studio S5; NGS data interpretation software: Ion Reporter/Oncomine Reporter.

IonReporter Software was one of the analysis and interpretation software used for the data obtained by NGS. This program provides us with information regarding the chromosomal location of the sequenced gene, the type of mutation (SNV-type mutations were selected - missense, frameshift/non-frameshift deletions and insertions, nonsense), the gene at which the mutation occurred, the mutation at nucleotides, amino acid substitution modification (in the case of missense mutations), as well as the targeted therapeutic indication for the identified mutation.

The Oncomine Reporter analysis program is a complex software that identifies, based on gene mutations, clinically significant biomarkers for which targeted therapies exist in the specified cancer type or in other cancer types approved by oncology authorities: FDA, ESMO, EMA, NCCN.

1.3. Methods for identification of tumoral neoepitopes

- Selection of alleles specific to the immunophenotype of the Romanian population and the global population;
- Identification of mutated proteins by NGS. Based on the single nucleotide variation (SNV) results of gene sequencing, the resulting amino acids were replaced in the protein sequences described in the NCBI database. By comparing the mutant protein

with the normal protein we identified the immunogenic regions using the Deimmunization algorithm from IEDB. Immunogenic regions (mutant compared to control) were tested using the IEDB algorithm for binding affinity to MHC class I molecules, transport to the endoplasmic reticulum by transporter proteins (TAP 1 and 2) and their proteasomal cleavage.

- Prediction of epitopes restricted to MHC class I. Prediction of HLA class I epitopes was performed using NetMHCpan, a prediction software based on ANNs, which allows the user to enter alleles in FASTA format for making the prediction. Among the parameters generated by the program is binding affinity, which was used to characterize HLA epitope-allele pairs. The threshold used was 500 nM. The second software used is IEDB tools. It does not allow the input of custom HLA allelic sequences in FASTA format, but provides in the documentation a list of reference HLA class I alleles that can be predicted to cover a large percentage of the global population (approximately 98.55%).
- The prediction of specific linear epitopes for MHC class I molecules was performed using an algorithm based on artificial neural network, online server NetCTL 1.2, which performs predictions for binding to MHC class I molecules, followed by sending the generated results to the VaxiJen v2.0 servers, ToxinPred, to identify non-toxic antigenic peptides. After performing epitope screening using VaxiJen v2.0 and ToxinPred servers, the resulting epitopes were tested for immunogenicity with IEDB server.

2. RESULTS

2.1. Identification of genic mutations in selected patients' groups

The present study proposed the identification and development of modern methods of diagnosis and therapeutic orientation in cancer (solid tumors) – next-generation sequencing (NGS).

Our research focused on the use of modern methods and techniques to achieve the listed objectives. The study was conducted on a group of 100 patients with solid tumors, aged between 20-79 years, of both sexes, who voluntarily presented themselves at the "Pius Brînzeu" County Emergency Clinical Hospital Timișoara - OncoGen Center. The investigations were carried out exclusively for research purposes on the samples of patients (paraffin blocks with tumor tissue) diagnosed with cancer by conventional methods.

To carry out Next-Generation Gene Sequencing (NGS), we followed several steps:

Sample preparation consisted of the extraction and purification of the genetic material (DNA) required for sequencing. The genomic library of the samples was subsequently performed, for which the genetic material was fragmented and a genomic library was created by adding specialized adapters to the DNA fragments to prepare them for sequencing. NGS sequencing was performed using the genomic library and allowed the simultaneous sequencing of thousands or even millions of DNA fragments, providing extensive genomic coverage. Sequencing data analysis was performed using specialized algorithms and bioinformatics software (IonReporter and Oncomine Reporter). This

includes the identification of gene mutations, copy number variations (CNVs), single nucleotide variations (SNVs), gene rearrangements and other tumor-associated gene changes.

The following categories of parameters were identified:

- Frequent somatic mutations, which are present in a significant percentage of patients with a certain type of solid tumor. These mutations were quantified for each type of tumor included in the study, in the 5 groups of patients (lung, breast, digestive, genitourinary and other types of solid tumors), being point mutations, deletions, insertions or rearrangements genetic (frameshift and non-frameshift), affecting certain genes known to play an important role in tumorigenesis.
- Allelic frequency, which indicates the proportion of tumor cells carrying the mutation in relation to normal cells in the tumor sample; the allelic frequency gave us information about the prevalence level of the mutation, being used to assess tumor genetic heterogeneity and subclonality.
- Localization of mutations, which gave us useful information to identify the affected gene regions and the involved exonic regions.
- Changes in the expression of proteins associated with each gene.

By combining this information, the analysis of the results obtained by NGS gave us a more complete and detailed picture of the genetic and molecular changes associated with each type of solid tumor, identifying common mutations in all the groups studied: TP53 p.(P72R), PIK3CA p .(I391M), KDR p.(Q472H), APC p.(A1582T/P) and NOTCH1 p.(V1578del) (Figure 2). These data can be used to guide the selection of personalized therapy, identify new therapeutic targets, and develop more effective treatment strategies.

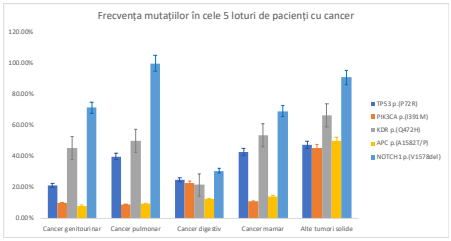

Fig. 2. Common mutations identified in the 5 groups of patients with solid tumors

2.2. Prediction of common neoepitopes in solid tumors for identification of immunotherapeutic strategies

After the NGS analysis and identification of mutations and molecular changes in tumor samples of patients from the 5 groups, the prediction of neoepitopes presented by tumor cells in the context of MHC class I molecules was performed (Figure 3). The MHC class I molecules most frequently represented in the population around the world and in Romania were selected and epitopes of 8-11 AA from the mutated peptide sequences of

the TP53 p.(P72R), PIK3CA p.(I391M), KDR genes were selected p.(Q472H), APC p.(A1582T/P) and NOTCH1 p.(V1578del). These epitopes will be recognized by the immune system as non-self, in the context of presentation with MHC class I molecules, resulting in the destruction of tumor cells presenting such changes. The following epitopes were identified, which may become therapeutic targets in the context of solid tumors: for the KDR protein - HLA-B*35:01-QAVSVTNPY; TP53 - HLA-A*02:01-RMPEAAPPV; PIK3CA - HLA-B*18:01- NEWLNYDIY.

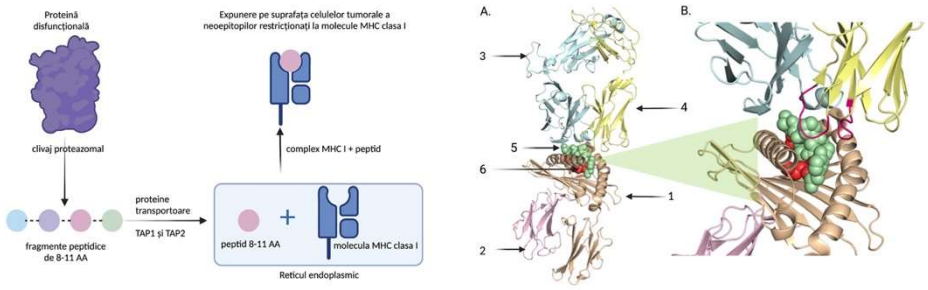

Fig. 3. Presentation of neoepitopes in the context of MHC class I molecules

CONCLUSIONS

This paper had 3 major research objectives, the activities and results obtained were carried out to fulfill them.

1. 100 paraffin-embedded tumor tissue samples from patients with solid tumors of different morphopathological types were analyzed by next-generation gene sequencing (NGS), divided into 5 groups: lung cancer, genitourinary cancer, breast cancer, digestive cancer and other types of solid tumors;
2. The somatic gene mutations were identified through the analysis of the sequencing data, which was carried out with the help of specialized algorithms and bioinformatics software (IonReporter and Oncomine Reporter), these changes being copy number variations (CNV), variations of single nucleotide polymorphisms (SNVs), point mutations, deletions, insertions, gene rearrangements and other gene alterations associated with solid tumors.

The study was conducted on a group of 100 patients with solid tumors, aged between 20-79 years, of both genders, who voluntarily presented themselves at the "Pius Brînzeu" County Emergency Clinical Hospital Timișoara - OncoGen Center was carried out in exclusive research purpose on paraffin-embedded tissue samples of patients diagnosed with cancer by conventional methods. The genetic material analyzed was represented by DNA, which was isolated, purified, quantified and used in the preparation of sample libraries, followed by next-generation gene sequencing (NGS) using a Hotspot panel, which identified mutations in 207 gene regions key to tumor development. By combining the parameters generated by the analysis of gene sequencing data, we identified common

somatic missense mutations in all studied groups: TP53 p.(P72R), PIK3CA p.(I391M), KDR p.(Q472H), APC p.(A1582T/P) and NOTCH1 p.(V1578del). These SNV-type mutations could become useful in personalized anti-tumor therapies.

The parameters used to identify common somatic mutations, frequently encountered in all groups of cancer patients studied were: frequent somatic mutations, which are present in a significant percentage of patients with a certain type of solid tumor; allelic frequency, which indicates the proportion of tumor cells carrying the mutation relative to normal cells in the tumor sample; localization of mutations, which provided us with useful information for identifying affected gene regions and involved exonic regions; structural and functional changes in proteins resulting from the transcription/translation process at the level of mutated genes. These data can be used to guide the selection of personalized therapy, identify new therapeutic targets, and develop more effective treatment strategies.

3. Based on prediction methods for the binding and activation of CD8+ cytotoxic T lymphocytes in the anti-tumor immune response, the following linear neoepitopes restricted to MHC class I were identified: for the KDR protein - HLA-B*35:01-QAVSVTNPY; for the TP53 protein - HLA-A*02:01-RMPEAAPPV; for PIK3CA protein - HLA-B*18:01- NEWLNYDIY. These neoepitopes could be used in anti-tumor immunotherapies in solid tumors. The current thesis makes an important contribution to the establishment of precision molecular diagnosis in solid tumors and to the identification of new molecular targets for personalized immunotherapies.

PERSONAL CONTRIBUTIONS

In this paper I have contributed in all the experimental steps that were carried out to achieve the objectives. Thus, I participated in:

- Elaboration of the informed consent form for cancer patients enrolled in the study; establishing the eligibility conditions of cancer patients/samples included in the study;
- Taking tumor tissue samples embedded in paraffin, isolating, purifying, quantifying the DNA and storing it at temperatures of -20^{o}C for later use in gene sequencing procedures;
- Preparation of sample libraries in the automatic system IonChef – IonTorrent; Automatic loading of libraries on chips and next-generation gene sequencing (NGS) with IonTorrent Gene Studio S5;
- Analysis of gene sequencing data and generation of analysis reports in IonReporter and Oncomine Reporter programs;
- Analysis of gene mutations identified for each patient and within each group of patients with solid tumors: lung, genitourinary, breast, digestive cancers and other types of solid tumors; generating statistical data using Microsoft Office tools – Excel;
- Identification of genes/mutations common to all groups of cancer patients – KDR, PIK3CA, TP53, APC, NOTCH1;

- Use of NetMHC, NetCTL and IEDB programs for the prediction of neoepitopes restricted to MHC class I molecules and their presentation by tumor cells in order to induce an anti-tumor immune response;
- Identification of candidate neoepitopes targetable by specific binding and activation of cytotoxic T lymphocytes: QAVSVTNPY (KDR), RMPEAAPPV (TP53) and NEWLNYDIY (PIK3CA).

FUTURE RESEARCH DIRECTIONS

Next-generation sequencing (NGS) is not only an accurate method of molecular diagnosis in cancer, but may be useful in identifying new molecular targets for anti-tumor therapy/immunotherapy.

In this work, we presented the technique to identify neoepitopes presented in the context of MHC class I on the surface of tumor cells, which become targets for cytotoxic T lymphocytes (CD8+) in the anti-tumor immune response.

This concept can be extended to the identification of neoepitopes presented in the context of MHC class II, which will lead to the activation of T helper lymphocytes (CD4+), with an anti-tumor effect directly through the secretion of specific cytokines (for example, IFN-gamma) or indirectly, through additional activation of the innate immune response (through phagocytic cells – macrophages or cytotoxic cells – NK) and co-activation of cytotoxic T lymphocytes. Also, within the immunotherapies generated by these methods, epitopes restricted to MHC class I (8-11 amino acids) can be coupled with epitopes restricted to MHC class II molecules (13-15 amino acids), specific for each mutated protein in part or in combination, from several such proteins, resulting in a synthetic long peptide (SLP) compound of 30-35 amino acids, which can be administered as specific and personalized immunotherapy to each cancer patient.

Another direction of future research is the identification of mutations by NGS at the level of genes that are translated into mutant transmembrane proteins, which will generate conformational neoepitopes targetable by another component of the innate immune system, namely B lymphocytes and antibodies (immunoglobulins), specific anti-tumor substances synthesized by them.

Last but not least, to the extent that the identified conformational neoepitopes are unique and specific to tumor cells, they may become the target for chimeric antigen receptor T cell immunotherapies (CAR-T).

Also, since intracellular signaling pathways in tumor processes are redundant and synergistic, anti-tumor therapies should be associative, comprising conventional therapies, immune system disinhibition therapies (check-point inhibitors) and targeted, personalized therapies, identified by NGS.