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Review

The Application of Next Generation Sequencing in Phage Display: A Short Review

Ali Aghebati-Maleki[1], Behzad Baradaran[1,2], Khalil Hajiasgharzadeh[1], Babak Bakhshinejad[3], Leili Aghebati-Maleki[1*]

[1]Immunology Research Center, Tabriz University of Medical Sciences, Tabriz, Iran
[2]Department of Immunology, Faculty of Medicine, Tabriz University of Medical Sciences, Tabriz, Iran
[3]Department of Molecular Genetics, Faculty of Biological Sciences, Tarbiat Modares University, Tehran, Iran

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Abstract

Phage display is a method of studying protein-protein, protein-peptide, and protein-DNA that uses bacteriophages to bind proteins with genetic information that encodes them. Phage display is a state-of-the-art technology for the production, identification, and engineering of fully human antibodies. Thus, many antibodies have been delivered to the medical community for research and clinical applications. In addition, next-generation sequencing to change their perspective on the mechanisms of human disease and has blended both clinical and basic research. Recent molecular biology techniques have greatly benefited from the development of this technique. Next generation sequencing has created a potential and reliable platform for the development of therapeutic monoclonal antibodies, providing an unprecedented insight into library diversity and clonal enrichment. In this study, we will briefly outline the applications of next generation sequencing in the biological sciences.

Introduction

In 1985, Smith devised a technique whereby researchers could study the interactions between billions of different peptides with their specific ligands. Phage display is a method of studying protein-protein, protein-peptide, and protein-DNA that uses bacteriophages to bind proteins with genetic information that encodes them.[1] This technique was then developed to express proteins such as antibodies. The relationship between genotype and phenotype in this technique, resulting in the screening of large protein libraries.[2] In this way, they can be screened and amplified in a process of *in vitro* selection. The most commonly used bacteriophages in phage display are M13 and fd filament phage. The principle of this technique is based on that filamentous phages can express small peptides as part of their surface proteins. Phages have several surface proteins in their structure such as: pVIII is the main coat protein of Ff phages and peptides that can be fused to pVIII are 6-8 amino acids long. The size constraints appear to be less applicable to the structural barrier caused by the added segments. pVI and pIII, which contain 5 copies at the end of the phage, and pIX, pVII, which correspond to five copies at the other end.[3] To accomplish this technique, it is sufficient to clone only random sequences of DNA into the phage genome in a suitable location among one of these genes. Then, this recombinant phage expresses the peptides in part of its surface proteins according to the location of random fragments of DNA in the genome. Phages whose displayed peptides bind to the target are captured, whereas the unbound or weakly-bound phages are removed. Repeating this operation for several rounds provides phages with a high affinity for the target ligand. Phage display is mainly used for high-throughput screening of protein interactions. By placing the large number of DNA fragments in the pIII or pVIII, we can create a library which, can be isolated the target proteins.[4] By fixing the DNA or target proteins on the appropriate affinity matrix, the phages can be bind to the protein fixed on the matrix surface remain, while the other phages will be removed by washing. The phages are recovered and then propagated by infecting bacterial host cells. Screening of phage display libraries to enrich the peptides that specifically bind to the target is accomplished through a dependency selection strategy called biopanning. Phages that are separated at the last stage of biopanning enter the bacterium for proliferation and the amplified phages are used for screening. Screening is also performed to separate more specific clones from the enriched phages. ELISA plates coated with specific antigens are used for screening.

*Corresponding Author: Leili Aghebati-Maleki, Email: leili_aghebati_maleki@yahoo.com

In 2007, a new way of sequencing DNA known as Next-generation sequencing[5] has been introduced and these technologies dramatically reduced the cost and time required for genome sequencing. For example, the human genome project took five years and cost over $5 billion but today by the use of the Next-generation sequencing[5] method, sequencing of the entire human genome cost about $5000 within a day. In fact, sample preparation with this technique is very faster than prior methods. The machine depended on a method called capillary electrophoresis that used Sanger's chain termination method without needing a gel. DNA sequencing is performed simultaneously with its synthesis from single-stranded template DNA. In other words, in these techniques, the binding of each type of nucleotide to the growing DNA chain is examined. Next-generation sequencing uses platforms that can fix and then analyze millions of pieces of DNA in separate positions.[6] As a result, several genomes can be sequenced in less than a week. These techniques are also very flexible and can be applied to all types and sizes of the genome, from viruses to humans. As mentioned above, these techniques make it possible for researchers to study thousands of genomes, find the differences in the nucleotide sequence of people around the world, and manifest the mutations that increase the risk of diseases ranging from cancer to autism.[7] Next generation sequencing techniques have also enabled the genomic sequencing of extinct species such as Neanderthals and mammoths.[8] It also makes it easier to understand the molecular basis of key evolutionary events in the phylogeny of tree life and the study of the biological system at a level of the nerve before possible. In addition, the technology that produces data that can be readily available without the need of a bioinformatics specialist is very profitable in various branches of biology and medicine.[9] With this brief introduction and definitions for each topic separately, we will attempt to briefly refer to the applications of these two approaches in biomedicine that help each other in the medical science progression.

Phage display applications

The launch of this method was described by Smith et al in 1985 in which they first characterized the external polypeptide screen on the surface of the filament phage particles. Over the past decade, significant progress had been made in the techniques and applications of phage libraries.[10] In addition, various screening methods have been devised to help the isolation and characterization of peptides that are bound to multiple molecules *in vitro*. In this section of the article, we will briefly outline the applications of phage display in the biological sciences.

Protein-protein interaction

Over 80% of cellular proteins may interact with other intracellular proteins in complex ways, and their interactions are controlled and regulated by several mechanisms. Phage display has been used in many investigations that study the interaction between two proteins.[1] This method can be combined with mutational analysis to provide fast and reliable approaches for identifying residues involved in protein-protein interactions. Phage-displayed random peptide libraries are a valuable screening resource for identifying novel reactive partners between proteins.[11] For example, phage display experiments can predict the interaction between the proteins carrying the bacterial membrane of TonB and BtuF and identify potential binding residues on each of these proteins. The peptides displayed on the phage are identified by binding to target candidates like TonB or BtuF with high affinity called biopanning. Sequence comparison of selected high-affinity peptides with TonB or BtuF amino acid sequences is performed with the MATCH program in the RELIC (receptor ligand contacts) and MatchScan bioinformatics model.[12]

Specificity of enzymes and inhibitors

Phage display is used in the study of enzymes to determine substrate specificity as well as to develop the design of both allosteric regulators and active site inhibitors.[13] It can also be used for the selection of enzyme mutants to study their mechanisms of action. Therefore, filamentous phage resistance to a wide range of proteases can be used as a substrate for the identification of diverse proteases. In order to isolate the protease resistance phage from the solution or to stabilize the phage before exposure to the protease, a random peptide library with a high affinity labeled N-terminal epitope is used for this purpose. The labeled fragment may be used to determine the affinity and specificity of protease for the phage encoding specific substrate by ELISA. This basic approach can be adapted to a semi-automated platform for general screening. In addition to selecting the substrate specificity, folded proteins are resistant to proteolytic cleavage when compared with unfolded proteins. Such choices may relate the protein sequence to its structure and may also help to engineer proteins with more folding and stability.[14]

Antibodies

Phage display of antibody fragments has been successful in generating target-specific antibodies that can be useful in a variety of applications such as proteomics, drug delivery, as well as intracellular process analysis.[15] The main advantage of using phage display for engineering antibodies is its rapidity and no need to immunize animals, especially humans. Naïve phage display antibody libraries can be derived from a set of recombinant V genes of non-immunized donors and synthetic antibody phage libraries by completely introducing rearranged V gene fragments into the germline complementarity-determining regions (CDRs). Naïve libraries with natural CDRs or synthetic libraries with artificial diversity in the V gene of CDRs can then be screened against more antigens including non-immunogenic molecules and protected species.[16] The advantage of this method is the screening of a naïve antibody library for the selection of specific antibodies

for each of the 20 human SH2 domains which, have a three-dimensional structure with a 20%–89% common sequence. Phage display is also used to isolate intrabodies that are penetrated the cell to access their intracellular targets.

Epitopes and mimetics

Phage display is an inexpensive and fast method to determine the antigen epitopes that interact with specific antibodies. Identification of epitopes is essential in the development of a vaccine, immunotherapy, and diagnostic strategies. Phage display peptide libraries can identify important residues within a continuous epitope involved in antibody binding.[17] Because continuous linear epitopes have 6 amino acids in length, screening libraries may select peptides that are precisely identical and cross-linked to the primary structure of epitopes. Random peptide libraries were applied to the epitope analysis by synthetic oligonucleotides or gene fragments. Gene-fragment phage display libraries are useful in identifying epitopes that have long structures. Phage display has been employed to identify mimetic peptides, which are discontinued epitopes. There is not any similarity between the linear sequence of the antigen and mimetic peptides. There are several analytical tools for simple epitope mapping based on selected mimetic sequences and 3D antigen structure. The phage display peptide library can also identify imitated carbohydrate and lipid antigens with low immunogenicity profiles. The mimetic peptide can couple with carrier proteins or can being a polymeric scaffold which, they display anti-pregnancy, anti-allergenic, and cancer vaccines properties.[18]

Receptors and G proteins

Phage display is used to identify agonists and antagonists to explore the structure and function of the receptor. Peptide libraries can be screened to bind to functional extracellular domains containing receptors for natural ligands. Selected peptides that recognize the receptor binding intermolecular interface can weaken its interaction with the natural ligand.[19] Functional and structural properties of superfamily receptors that bind to similar natural ligands can be characterized by high-affinity selected peptides that are specific to each member. Phage display is also selected for the screening of peptides bind to G protein-coupled receptors, where it is difficult to purify functionally correct folded extracellular domain receptors. Antibodies specific to the known receptor-ligand can also identify some peptide molecules that mimic epitopes (named mimotopes) from the phage display library. Selected mimotopes are used to study the mechanism of ligand-receptor interaction and allows the development of protein agonists and antagonists.[20] Also, receptor antagonists can be selected by binding to the specific receptors and thereby prevent their interaction with the other receptors. An example of that is the discovery of peptidic antagonists of IGF-1 function. Around 22 phage displays, random peptide libraries with the diversity display scaffolds in a polyvalent format were pooled and screened for IGF-1 binding.[21] The selected phage clones were bound to IGF-1 and the phage display library was designed based on the motifs of the protected clones. The growth of the subunit library in the monovalent format resulted in a peptide that inhibits the activity of two IGF-1 cell surface receptors and two IGF-1-binding soluble proteins with a small amount. The phage display method is used to determine the biological affinities of proteins that bind to active drug compounds such as SB-236057, taxol, and FK506.[22] The identification of these proteins helps to determine the mechanism of action of these compounds.

Next generation sequencing applications

Sequencing techniques are able to assist biologists in a wide range of applications such as cloning, finding pathogen genes, and comparative studies. Next generation sequencing techniques with high throughput open a new viewpoint in biomedical research. Also, the next generation sequencing devices have created a huge revolution in genetics and they were able to sequence the human genome at a very low cost within a day with high quality.[23] Until a few years ago, the Sanger method for sequencing was used in 1977. But with the advancement of science, the sequencing method has undergone a major change, and nowadays, it is possible to determine the genomes of different species at high speeds and very low cost by next sequencing techniques.

The application of NGS in animal studies

Next-generation sequencing has a potential role in animal biotechnology subsets and veterinary research ranged from a fundamental change in traditional animal reproduction to the identification of a very small gene variation.[24] Recent molecular biology techniques have greatly benefited from the development of this technique.[25] These can be used in evolutionary research in animals, epigenetics, metagenome sequencing, ancient DNA, genomic diversity, and SNP/CNV discovery, as well as in the selection of good flocks for meat production, animal reproduction, food safety, nutrition, and finally in the production of transgenic animals.[26]

The application of NGS in human health

Next-generation sequencing to change their perspective on the mechanisms of human disease and has blended both clinical and basic research. The basic science research is either directly carried out by the NGS for sequencing new varieties or by the information generated through the NGS for experimental purposes.[27] Clinical trials include high-throughput genetic testing and related clinical genetic follow-up. We will briefly mention them below, where NGS has important impacts.

Cancer research

Next-generation sequencing[5] has changed cancer research areas. Since cancer research has been traditionally concerned with the fact that there are no specific mechanisms for different types of cancers, we need to analyze a large number of genetic variations in the human genome that may be correlated with cancer phenotypes.[28] A large number of cancers (as well as healthy ones) require a study to compare their genomes with multiple genetic targets. With the introduction of next generation sequencing, the latter has greatly benefited from the fact that multiple genomes can be sequenced in a few weeks. However, the cost of sequencing target DNA, especially exome sequencing reduced significantly. This facilitates the analysis of genetic diversity involving millions of SNPs associated with a particular phenotype in the Genome-Wide Association Study. Currently, international cooperation was done underway to catalog cancer mutations.

Genetic disorders

Recent efforts have shown a significant opportunity to use next generation sequencing to detect genetic disorders such as hypertension, diabetes, and Mendelian disorder diagnosis and it is feasible for clinical applications economically. This technique has significantly modified the disease-associated mutations and genetic alterations. The NGS technique has increased the capacity of researchers to analyze the large panels of genes for one individual. In recent years, technical advances in the NGS, especially target enrichment methods, have led to the identification of genetic diversity for more than 40 rare disorders. Even the identification of mental genetic disorders has been improved by the use of NGS.[28]

Human microbiome

Harmful and beneficial microbial organisms are closely associated with the human body. These organisms not only are mainly bacteria but also include other organisms such as yeast, single-cell eukaryotes, viruses, and more.[29] Most of these organisms have not been successfully cultivated, identified, or identified. These organisms can form an important site of human bodies include the colon, stomach, vagina, skin, hair, etc. In 2008, the Human Microbiome Project (HMP) was launched by the US NIH to identify and characterize the microorganisms found in healthy, and diseased individuals. With the help of next generation sequencing, we can identify newly colonized germs using metagenomic sequencing or by 16S rRNA gene amplification sequences from mixed populations or whole-genome sequencing.[30]

Pre- and Post-natal diagnosis

One of the valuable applications of next generation sequencing technology is molecular genetic testing in pre- and postnatal diagnoses. Traditionally, invasive genetic sampling techniques have been used for sampling and examining conventional chromosomal abnormalities that were both dangerous to the mother and the fetus. Fetal aneuploidy study by next generation sequencing in high-risk pregnancies is a good and reliable method and has been used successfully to detect chromosomal aneuploidy of fetal DNA from DNA fragments in the mother's plasma (cell-free DNA fragments). The advantage of next generation sequencing over traditional methods of prenatal diagnosis is that it is a non-invasive technique with high sensitivity and specificity.[31]

Infectious diseases

The next generation sequencing method has become a central component of infectious and genetic disease research and diagnostics because of the reduction of DNA sequencing costs. This method is first used in viral research to explore the deep sequencing influenza virus genome, to identify the genetic diversity and evolutionary pathways of HIV within the host. Next generation sequencing is also used to monitor antiviral drug-resistant mutations. Next generation sequencing has been used as a powerful tool for the study of bacterial genomics, viral dynamics, host response, and other biological aspects of infectious diseases that were previously unavailable.[32]

Personalized medicine

Each person is genetically different. Therefore, susceptibility to different diseases, infections, and disorders are variable. In addition, every person needs different treatments and management of diseases. In order to make the right decision, a person's genetic structure must be identified. Our genome contains information that indicates the risk of a number of hereditary diseases. Next-generation sequencing technologies can provide a range of information about the different types of disease-causing changes in individuals that are needed in a short period of time to screen patients for pre-clinical tests or clinical diagnoses. It is also useful in identifying and developing panels for biomarkers.[33]

The application of next generation sequencing in phage display

As mentioned above, phage display is a state-of-the-art technology for the production, identification, and engineering of fully human antibodies. Thus, many antibodies have been delivered to the medical community for research and clinical applications.[34] A typical antibody phage display experiment consists of three steps: 1) Generation and display of an antibody library such as the Antigen Binding Fragment (Fab) library or the Single-Chain Variable Fragmen library. 2) Enrichment of antigen-binding antibodies by multiple binding selection rounds and amplification. 3) Recovery of antigen-specific antibodies from phage selection pools. The Sanger sequence is commonly used at each of these steps to decode the sequence of recovered antibody fragments from the phage pools. It requires the isolation of individual

phagemids from phage pools and is practically restricted to sequencing a few hundred clones routinely. Since there are about one million sequences in each output phage display, if one wants to analyze all the sequence variations present in the selected phage outputs, the use of NGS sequencing is essential. With next generation sequencing, all phage pools can be sequenced and can easily reach from one million to one billion sequences.[35] The development of antibody display technology such as phage, ribosome, and yeast has made a fast choice of Binders from a variety of libraries. These technologies bypass the use of animals and cause the enrichment of connectors within days to weeks. The power of in vitro selection technologies relies on the direct physical linkage between the phenotype (the displayed antibody) and the genotype (the second variable of the antibody genes), which allows the identification of the linkers by their sequencing of coding genes. Typically, in order to choose the best antigen-binding antibody, multiple rounds are required, either by bonding to a solid substrate or by cell stratification. In most cases, the last rounds of selections are dominated by a number of clones that are subsequently characterized for the affinity test. Such colony dominance reflects the actual choice for high-affinity binding sites for antigen but reflects other attributes such as more expression or representation.[36] As a result, high-affinity clones may be present in low amounts and may not be detectable by using traditional screening techniques such as ELISA. Recent advancements in DNA sequencing technologies and computing power over the past decade led to significant reductions in the cost of sequencing and also have made data analysis easier. Although genomic applications such as whole genome sequencing, transcriptome sequencing, and Epigenetics were used early, next generation sequencing technology[5] in other fields including functional and basic immunology is being increasingly used nowadays. This includes sequencing of light and heavy chain antibody tanks, antigen-specific B cells, as well as T cell receptors and antibody display tanks.[37] While most NGS platforms are designed specifically for short reads, they are also rapidly expanded, and also reading length and depth are expanding. Here we briefly review recent advances in NGS technology and its key application in phage display and other selected technologies *in vitro*.

The strategies for next generation sequencing antibody display repertories

Traditionally, antibody display libraries are analyzed by isolating one hundred to thousands of clones with a combination of Sanger sequencing. Although this method is sufficient to identify dominant clones after selection, the data obtained show only a limited picture of the actual library diversity.[38] Comparing NGS methods with providing more than ten million sequences (10 000 times Sanger sequencing) allows us to see a wide range of library diversity. One of the challenges in using NGS to analyze antibody selection systems is the size of the coded genes.[39] The smallest antibody fragments have a range between 400-300 bp in length, whereas the Fab and scFv antibody fragments each have a range of 700 to 800 bp to over 1500 bp. While the next generation sequencing technology[5] is adapted for short reads (less than 100 bp), however, many platforms can read up to 300-400 bp. Long sequences can be performed using paired-end readings, this method is particularly useful for scFv formats and providing the sequences of multiple CDR domains of VH and VL domains. In addition to analyzing the sequences of larger antibody fragments, some studies have focused only on the relatively short VH CDR3 repository sequences. Using next generation sequencing requires special attention to sequencing errors. Inevitably, DNA replication leads to polymerase errors depending on the circumstances. Although polymerase error rates are typically low, the errors are visible in large NGS data containing billions of bits. Unique molecular identifiers are added to the primers to help identify sequencing errors and PCR during the first two cycles of PCR amplification.[40]

Application of NGS in design validation and the analyses of Naïve antibody libraries

When producing antibody display repertories, either synthetically or derived from immunized animals, it is essential to verify the clone diversity of the library before selection. Numerous studies have indicated that the use of NGS to measure diversity can be used to support the design of displayed libraries.[34] High-throughput sequencing methods are not limited to human sequences. Recently, next generation sequencing has studied the diversity of Fab Rabbit libraries. Next generation sequencing is also used to accurately determine the size of a library. A study for this purpose has created a VH library derived from a donor.

Application of NGS in affinity maturation and epitope mapping

Next generation sequencing can also be used to select clones with high affinity.[41] A study used next generation sequencing to direct scFv fragment maturation against the ErbB2 receptor to achieve a final 25pM affinity (which resulted in a 158-fold enhancement of wild-type and natural affinity). This is driven by a structure-based design in which, individual CDR regions (except VL CDR2) were selected against ErbB2 antigens randomly, and exposed by NGS before and after panning. This results in the enrichment of motifs with new sequences in diverse CDR regions except for VH CDR3, which is enriched in the wild-type motif. Then most CDR replacements are combined to produce a second library that is selected against the target.[42] This results in an increased affinity from 300 to 25 pM compared to wild-type affinity of 4 nM.[43]

Conclusion

The next generation sequencing technology has created

a potential and reliable platform for the development of therapeutic monoclonal antibodies, providing an unprecedented insight into library diversity and clonal enrichment. Although the current NGS platforms are not designed with respect to antibody libraries, these technologies are currently at a stage where one can gain awareness of all selection processes with a unique sequence. In addition, with the advancement in sequencing technologies, the depth and length of the readings are constantly improving, for example, the PacBio platform provides approximately seven times more sequences than the older RS II system while retaining its long reading capability. Also, nanopore systems such as MinIOn offer simultaneous sequencing of DNA in combination with very long reads. As a result, with the development of NGS technology in a rapid state, the importance of sequencing analyzes of phage antibody-display libraries is likely to increase.

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Authors' Contribution

Ali Aghebati-Maleki conceived the idea and provided a draft of the manuscript. Behzad Baradaran participated in the literature survey. Khalil Hajiasgharzadeh and Babak Bakhshinejad provided inputs for the design and final edition of the article. Leili Aghebati-Maleki critically revised the manuscript. All authors read and approved the final manuscript.

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Data sharing is not applicable to this article as no datasets were generated or analysed during the current study.

Ethical Approval

Not Applicable.

Conflict of Interests

The authors declare that they have no conflicts of interest.

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