REVIEW ARTICLE

Conventional and Nano- Antibodies and Their Future Uses

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Abstract

Antibodies are the end products of the humoral immune responses and are produced exclusively from plasma cells. The natural structure is glycoprotein also called immune protein because their function is defence by specialization. The main functions of antibodies are opsonisation, neutralization, agglutination, precipitation and complement activation "fixation". Conventional antibodies consists of two heavy chains and two light chains and can divided into five Classes, based on diversity in the amino acid sequences in the constant region of heavy chains. Also antibodies can be classified into three different classes including polyclonal, monoclonal and recombinant antibodies dependent on immunotechnology applications. In addition to conventional antibodies in 1993 Hamers-Casterman et al, discover antibodies contain only two heavy chains, and produce from few animal species. These review abductors on these types of antibodies and the extent of independent thinking to be used in the treatment of incurable disease such as cancers.

Keywords: Antibodies; Immune protein; Light chain, Heavy chain; Conventional antibodies; Nano-antibodies.

Conventional Antibody

Conventional antibodies (whole size) are a glycoproteins, secreted by differentiated B-cells to named plasma cells and response to foreign antigen "immunogenic" [1], these antibodies are named immunoglobulin [2], which act as a critical part of the humoral immune response by recognizing and reacting specifically with foreign bodies [3], the primary function of antibodies is to bind specifically to an antigen and elicit an immune response, and destruction of extracellular pathogens, and prevention against the spread of intracellular infections, and protecting the host from infection.

Conventional Antibody Structure

Conventional immunoglobulin G (IgG) consists of two heavy chains and two light chains. The heavy chains consist of a constant region (C_H), organized into three constant domains (C_{H1}-C_{H3}) and one variable domain (V_H). The C_{H1} and C_{H2} domains are linked by a hinge region. The light chains are composed of one constant (C_L) and one variable domain (V_L). The paired V_H and V_L domains are responsible for antigen binding; each antibody (monomer or basic unit) has a Y-shaped unit [4]. The basic unit of a complete antibody consists of a four polypeptide unit containing two identical large heavy chains (50–70 kDa), and two same size of small light chains"25 kDa" [5] bound together by disulfide bonds [6], and the hinge region at the point of the Y structure has flexibility, each polypeptide chain consist of :

- Constant region "crystallizable region (Fc)"
- Variable region "antigen-binding region (Fab)"

Constant region is the same for all antibodies, and it's not recognized as antigen, but it's responsible for mediating effector functions such as antibody-dependent cell-mediated cytotoxicity (ADCC), antibody-dependent cellular phagocytosis, antigen presentation to the immune system, degranulation, complement-mediated lyses, and effectors function which triggers the elimination of antigen like complement activation and macrophage activation, while the variable region is responsible for recognized the antigens and its includes one variable region (variable light or V_L) of the light chain and one constant region (C_L) of the heavy chain [7, 8], and forms the antigen-binding region (Fab) of the antibody, where the complementarily-determining regions (CDRs) can be located [9]. The CDRs from the antigen-binding sites of antibody and recognized the antigen with specificity, the Fab part retains the antigen binding activity while the Fc part form most of the constant region of the heavy chains [10].

Antibodies Classes

The antibodies are divided into five isotype, based on diversity in the amino acid sequences in the constant region of heavy chains [11] are IgG (Gamma heavy chains), IgM (Mu heavy chains), IgA (Alpha heavy chains), IgD (Delta heavy chains) and IgE (Epsilon heavy chains). The main functions of antibodies are opsonisation, neutralization, agglutination, precipitation and complement activation "fixation" [12]. As described above classes of antibodies, the IgM is the first antibodies to be produced in primary response; it's expressed on the B cell and in a secreted form (pentamer) with very high avidity, and it's responsible for activation of phagocytosis [13]. An IgA class antibody is mainly found in mucosal surfaces such as the gut, respiratory tract and prevents colonization by pathogens [14]. IgE is an important of antibodies contributed in defense against parasites and play a critical role in allergic diseases [15], while IgD isotype is found just in a little amounts as antibody but its function still unresolved [16] and finally IgG are with 70-75% of the total immunoglobulin the most abundant antibodies [15], the only antibody capable of crossing the placenta to give passive immunity to the fetus.

Antibodies Categories

Antibodies can be classified into three different classes including ploy clonal, monoclonal and recombinant antibodies [17].

- Polyclonal antibodies (PAb) are heterogeneous antibody mixtures that are derived from several plasma cell lines. Because polyclonal antibodies

comprise a complex of different antibodies that have numerous paratopes, they also have excellent properties for recognizing antigens [18, 19].

- Monoclonal antibodies (MAb) are a homogeneous antibody coming from a single B lymphocyte clone and produced in monoclonal antibodies format have a unique high specificity against a single epitope [19].
- Recombinant antibodies (RAbs) are antibodies generated using molecular biological methods. They are aimed to improve the stability, sensitivity, selectivity and immobilization properties in diagnostic applications [20].

Conventional Antibody Applications

Because of the high specify of antibodies against several antigens, the antibodies are used as a study instrument for target detection, helping in medical diagnosis and therapy, therefore the antibody is considered basically for a very broad range of application and then orientation to produce the monoclonal antibody (mAbs) and antibody fragments (parts)which have become a significant segment of industrial and academic research due to their proven efficacy, safety, and manufacturability profiles [21].

Monoclonal antibody production is an old immunological technique with great applications in the fields of biochemistry, immunology, biotechnology and applied biology among others [22], it is used in immunodiagnostic techniques as reagents to detection antigen of the causative agents or antibodies detection against the causative agents [23] like ELISA, western blot, immunodot blot, flow cytometer, immunohistochemistry, radioimmunoassay (RIA), microscopy (electron, fluorescence, confocal) and other biotechnology applications [22,24,25].

The monoclonal antibodies have rebellion cancer treatments [26],and it relies on small- molecules drugs that easily enter to tumor cells or monoclonal that connect to specific target on their surface and is use as a immunotherapies target different goals like the blockade of oncogenic pathways with subsequent effect on cells growth and apoptosis [27], the blockade of the formation of new blood vessels [28], the permutation of immune response against tumor cells [29], opposite polyclonal antibodies, the monospecificity of a monoclonal antibodies improve to targeting of a single epitope.

Despite the success of antibodies as treatment in patients but they has the disadvantages that their inability to penetrate into certain tissues due to their big size and heavy ~150 kDa [30], also the Fc domain will stimulate the body's immune response and its target bound molecules for destruction, which may be detrimental to the patient's health; resistance hard accessibility to target antigen and high cost have restricted them widespread application in patients, low stability; therefore, multiple studies are ongoing to develop new way for improving the specificity of monoclonal antibodies, and the cost-effective expression systems to overcome the limitations mentioned previously [31].

Nanobodies (Nbs)

In addition to conventional antibodiesH_2 L_2 -type IgGs [32], in the serum of Camelidae family like (Bactrian camels, dromedaries and llamas), also possess special IgG antibodies homodimeric H_2 -type antibodies which were called heavy chain-only antibodies (HCAbs) IgG [33], which as a part of the humoral immune response known as heavy-chain antibodies "HCAbs" [32,34]. These antibodies contain only two heavy chains, while the light chain and C_{H1} domain are absence, and lacking $C_{H1}$is necessary for anchoring the light chain in conventional antibodies [35, 36].

The C-terminal VH region derived from HCAbs its named variable domain (V_{HH}), also referred to single domain antibody (sdAbs) or " Nanobody®" a trade name of Ablynx" [37-39] which without VL. The hinge region joined directly to this domain, and the extended hinge region in heavy chain antibody (HCAbs) due to its high proline content, and promotes their flexible and stability the antibody [40,41]. In cartilaginous fishes like sharks, rays, and skates [42,43] are have heavy-chain antibodies (HCAbs) which formed two single-domains antibodies (V_{HH} or nanobody) are called 'immunoglobulin variable new antigen receptor (Ig_{VNAR} fragments).

In general the nanobodies have a molecular weight about 12–15 kDamuch smaller than conventional antibodies with molecular weight about (150–160 kDa) and even smaller than antigen binding site "~50 kDa"and single-chain variable fragments "~25 kDa" of conventional antibodies [44,45].

Expression of Antibodies in Endoplasmic Reticulum (ER).

The Camelidae family has three major subclasses of circulating γ immunoglobulins (IgG): IgG_1, IgG_2, and IgG_3.Immunoglobulin G_1 is a heterotetrameric antibody (i.e. two heavy chains and two light chains) while IgG_2 and IgG_3 are homodimers of only heavy chains (i.e. two heavy chains, while light chains and C_{H1} are absence). Immediately after translation, heavy and light chains combine together in the endoplasmic reticulum. The major ER protein is called heavy chain binding protein (BiP) [46]. A nascent heavy chainis bound by BiP immediately after translation and is retained until a light chain can replace BiP [5]. If a light chain is absent, the heavy chain/BiP complex is bound by E3 ubiquitin ligase complex and transported across the membrane to the proteasome [47]. BiP binds specifically to the C_{H1} segment of the heavy chain [48], and retains the heavy chain in the endoplasmic reticulum until BiP is exchanged for a light chain. Since C_{H1} regions are not present in antibodies belonging to the camel class IgG2 and IgG3, such antibodies cannot be retained by BiP and are exported.

The mechanism of removal of exon encoding C_{H1} during mRNA processing [35], camelids carry a nucleotide G to a point mutation that disrupts the consensus-splicing site at the 5′ end of the intron between the C_{H1}-hinge exons and provokes the elimination of the C_{H1} region from the messenger ribonucleic acid by splicing [49,50].

Characteristics and Advantages of Nanobodies

Nanobody (Nb) is a peptide chain comprising of (110) amino acid and forming one variable domain of heavy chain antibody IgG [51], the conventional IgG were present which contributed up to 75% of all serum in Camelidae family [34], the immunoglobulin IgG is divided into subclasses are IgG1,IgG2 , IgG3.

The immunoglobulin IgG1 is a conventional antibody with a molecular weight about ~150 kDa, while two other immunoglobulin fractions are non-conventional antibodies are IgG2 and IgG3; with molecular weight ~90 kDa. These nanobodies have similar affinity and specificity of conventional antibodies [52] to different antigens, but it has unique features [53] that are not accessible to conventional antibodies:

- Nanobodies have small size only tenth the size of conventional antibodies that lead to more effective permeability in tissues [45, 52], especially translocation through the blood-brain barrier [54], than of conventional antibodies.Because of their size in the nm range, hence the term 'nanobody' [45].The diameter and length of VHH crystals are only about 2 and 4.5 nm, respectively, making them the smallest functional antibody fragments known [55].

- Nanobodies more heat-resistant survive at a temperature 90 ^{O}C for an hour without loss of their ability to bind antigens [56, 57].
- Nanobodies are less lipophilic and more soluble due to the increase of net charge of the protein [58] compared to the conventional antibody (V_H) and may due to the hydrophilic amino acid substitution found in the region of framework 2.
- For conventional antibodies, it is established that upon antigen binding, the ADCC and complement-dependent cytotoxicity are triggered by the Fc region [59, 60]. These two mechanisms play a critical role in the process of tumor elimination, as they both stimulated activation of cell lyses, and hence apoptosis cascades [59], for this reason, it was proposed to extent nanobodies without an Fc region [60].
- Nanobodies easy eliminated reanly unlike whole antibodies [33, 44, 45].
- Nanobodies can interact with a greater number and different of epitopes than of conventional antibodies [52, 61], and opened a new window in breast cancer therapy [62].
- Nanobodies are easy to produce in bacteria [63], yeast like Pichia pastoris and Saccharomyces cerevisiae [64] or in plants [65], and enabling large-scale production at reasonable costs [66].
- Nanobodies are considered to be non-immunogenic due to their high similarity with human $V_H$sequences; these unique properties triggered numerous applications in fundamental research, diagnostics, and therapy [34, 45, 66, and 67].
- Nanobodies are capable of recognizing haptens and cryptic epitopes that are not accessible to conventional antibodies [68].
- Nanobodies can bind to their targets with a high affinity and can be produced by simple methods with high yields [69].
- Nanobodies also demonstrate high stability even on exposure to extreme conditions such as very low/high pH and temperature [70].
- Nanobodies are procured by cloning the VHHgene and panning by phage display [49, 71] by using M13 helper phage.
- Nanobodies consist of four framework regions (FR1, FR2, FR3, and FR4) which form the core structure of the immunoglobulin's domain, and three complementarily-determining regions CDRs [34], the longest being CDR3 [72] that are involved in antigen binding which plays a critical role in their stability and binding affinity [34,45,73,74], this due to possess an additional disulfide bridge linking the CDRs (CDR1 and CDR3) also CDR3 is longer than CDR3 in conventional antibodies and that lead to form finger-like structure that enter into cavities on the surface antigen [34,73,75].

Nanobodies Applications

Because of these unique properties of nanobodies triggered numerous applications in fundamental research, diagnostics and therapy [34, 45, 66, 67]. So far, fast and reliable in vivo diagnosis of cancer at an early stage of disease progression remains a major challenge. Furthermore, in cancer diagnostic tests, nanobodies besides diagnostic applications for in vivo imaging due to their fast blood clearance and good tissue penetration, nanobodies have proven useful as specific drug delivery vehicles in tumor targeting [76]. The small size of this particle can ensure delivery of hidden antigens to the cancerous tissue without effecting healthy cells while healthy cells stay unharmed. It was reported that a nanobody specifically recognizes lysozyme targeted tumors transgenic for lysozyme [77]. Further potential therapeutic areas

include inflammation [38, 76]. Nanobodies can be used also to mark cells for sorting by flow Cytometry or magnetic beads.

Nanobodies Diversity

The development of conventional antibody interacts of V_H specifically with the V_L domain together. In the absence of a V_L domain, these lead to cause unspecific aggregation of HCAbs. Generally, the heavy chain variable domain (both V_H and $_{VHH}$) is encoded by multiple gene segments [10], the V (variable) gene segment, the J (joining) gene segment and the D (diversity)gene segment [78]. During B cell differentiation, the gene segments of antibody is specifically legated in a process named VDJ recombination to form a whole heavy chain variable exon [79] in which the V gene segment encodes the CDR1 and CDR2, while the CDR3 is formed by the joining of the three gene segments. In order not for the amino acid substitutions which are specific for nanobodies (V_{HHs}), camelids heavy chains to arise from the same V genes as conventional antibodies [35]. Nanobodies (V_{HHs}) are encoded by different set of V segments (~40 V segments).

Display Technologies to Obtain Recombinant Monoclonal Antibodies

In addition to the aid DNA engineering, surface display has been commonly used to engineered and isolate peptides, antibody fragments of scFv, and a single domain for selection monoclonal antibodies and using for the clinical application. The main advantage of the in vitro display technology is the possibility to obtain antibodies to any kind of targets and epitopes because the construction of a naïve or synthetic antibody repertoire is not dependent on an in vivo immune response [80], these display technologies such as display of (DNA, bacterial, yeast, ribosome, mRNA and phage).

DNA display

DNA display technology is a simple technique compared with other fully in vitro display technologies, with fewer steps [81].This display technique is used for in vitro selection of peptide ligands from a large library of peptides displayed on their encoding DNAs [81,82]. The advantages of this technology not require the RNase-free conditions are not need for the selection step because of not need the reverse transcription step. While the disadvantage of DNA display technology devoid of a robust platform and knowledge in comparison to other in vitro display technologies.

Bacterial and yeast display

Cell surface display systems have been developed for different host microorganisms, including bacteria and yeast cells [83, 84]. Bacterial and yeast display is the other in vitro display technology, both of these techniques depended on fusing the gene of the binder library to respective surface proteins, the bacteria and yeast can be screened via flow Cytometry. The generation of nanobodies by bacterial fermentation is simpler, quicker and cheaper than conventional antibodies generation [85].

Bacterial display technology is uses bacteria cells like Escherichia coli, as a host for expression and engineering of antibodies fragments like single domain antibody [86, 87]. On the other hand, yeast display technology uses yeast cells to display exogenous peptides or antibody fragments on the cell surface of the host cell [88].

Ribosome and mRNA display

Messenger RNA and ribosome display technologies as appearing a revolutionary in vitro display platform to avoid cell transformation steps. Ribosome display including the in vitro transcription and translation of a DNA library; and independent of E. coli transformation. The characteristics of mRNA display and

ribosome display technology helps to build much larger libraries [89] than the other in vitro display technologies because it avoids the library size limitation of phage and yeast display technologies which rely on the efficiency. The other advantage of this technology is the extensive use of PCR that contributes to the provider of more diversity to the library by mutations. In addition, mRNA and ribosome display platform can be used for the selection of novel molecules, including antibody fragments, and for affinity maturation of antibodies [90].

Phage display

Phage display is a powerful and robust technology, it was the first technology introduced by George Smith in 1985, that allows display of Nbs and other Ab fragments with high affinity and specificity to several target antigen on the surface of filamentous bacteriophages such as M13 or f1 [91-93], which infects E. coli through the F pilus. Phage display facilities selection of proteins, peptides, or antibodies with suitable binding properties from a large collection of variants, so named phage display library. Individual phages comprise a defined binder on the surface and the respective gene within a phagemid inside the phage particle. Challenging this phage library with an immobilized antigen allows for in vitro selection of specific binders that can be amplified and identified by reinfection of Escherichia coli.

Bacteriophage

The most widely used are the Ff class (f1, fd and M13) which have shown to be 98% homologous. The filamentous M13 phage is most commonly used in phage display technique [94]. An M13 bacteriophage virus that infects only male Gram-negative E. coli (bear the F-plasmid) [95, 96], which encodes the F-pilus like (TG1, SS320, ER2738, or XL1-Blue E. coli). The infection is mediated by the interaction between F-pilus and g3p of the phage, compared with lytic phage T4 and T7 which lyses and killing the bacteria cell while the M13 bacteriophage infected the host cell without effected on it (continue to growth and divide).

The phage is 6.5 nm in diameter and 930 nm in length with a covalently closed single-stranded genome of 6400 nucleotides coding for 11 different viral proteins (pipit) present within the coat proteins [97]. Five of the 11 proteins (pIII, pVI, pVII, pVIII, and pIX) make up the flexible protein cylinder, 2 (pII and pX) are involved in single-stranded DNA (ssDNA) [98] replication and 3 are required for phage particle assembly. The length of the cylinder is composed of 2700 molecules of the major coat protein (pVIII) while the minor coat proteins (pVII and pIX) are found at one end of the phage particle with 5 molecules of both gene products. There are approximately 5 copies of the other minor coat proteins (pIII and pVI) at the other end of the phage particle. The packaging signal is located at the pVII and pIX end of the phage particle and this is the first region to be assembled. Such libraries are generated by genetic fusion of genes of interest to those of phage coat proteins (pIII or pVIII) [99] using a special phagemid vector which is then transformed in E. coli. the gene 3 protein (g3p) is most commonly used for displaytechnology [100,101].

M13K07 Life Cycle

The infection process is a multistep process involving the F pilus on the surface of the cell, and the phage comes in contact with the pilus of *E.coli*, it retracts drawing the phage particle into the cell. Pilus formation is at its most abundance at mid-exponential phase of growth and decreases with increasing growth profile. The outer membrane bacterial proteins (TolQ, R and A) are needed for translocation of the filamentous phage DNA into the cytoplasm and the translocation of the phage coat proteins into the cytoplasmic membrane.

The initiation of infection is occurs upon binding of the tip of the F pilus to the pIII phage protein. The pIII phage protein consists of 3 domains (N1, N2 and CT) separated by glycine-rich regions. Each domain is essential for bacterial infection. The N1 and N2 domains are in close proximity and N2 is responsible for binding to the F pilus. The N1 domain interacts with the bacterial membrane protein (TolA-D3) and the phage is retracted towards the bacterium surface where the major and minor phage capsid proteins are disassembled and the phage DNA is translocated into the cytoplasm. The viral (+) phage ssDNA strand enters the cytoplasm and the complementary (-) DNA strand is synthesised by bacterial enzymes. This replicative form (RF) is the template for transcription and phage protein translation [102]. The pII protein cleaves the intergenic region of the positive strand of the RF which acts as a primer for synthesis of a new viral strand by a rolling-circle‘ replication method continuing until 200 copies are within the cell. This continues until pV approaches a crucial level where it binds to newly synthesised viral ssDNA. The newly synthesised ssDNA-pV (800 nm in length and 8 nm in diameter) is assembled in the cytoplasm of the bacterium and the phage particle is subsequently secreted from the cell. This is then cleaved and used as a template for further phage propagation. Approximately 1000 phage per cell are produced in the first hour with 100 – 200 phage particles in subsequent cycles consideration of a vector for the display of proteins or peptides as coat protein fusions to pVIII or pIII is important.

In phage vectors the gene is inserted directly into the genome and expressed with multiple copies of displayed protein leading to polyvalent display. This causes the selection of lower affinity variants during the bio panning process. To avoid an avidity affect a monovalent phage display process was developed using a phage-plasmid vector (phagemid). This is a plasmid with a plasmid origin of replication and a phage-derived origin of replication. The vector is capable of producing large quantities of pIII-fusion proteins but cannot make phage. This is aided by the addition of helper phage (i.e. M13K07 and VCSM13) that supply all the phage enzymes and proteins needed for phage replication [103]. Such libraries are produced by genetic fusion of genes interest to those of phage coat proteins (pIII orpVIII) [104,105] using a special phagemid vector which is then transformed to *E. coli*. Phagemidsin both E. coli and a phage contain a specific replication origin and a selection marker resistance. However, phagemids lack genes that responsible for replication, packaging, as well as coat proteins, therefore dependent on helper phages which are co-infected with the phagemids into host bacteria enables the phage to encode the required proteins for generating complete phage particles [106], leading to release of phageparticles necessary for encoded protein on their tip (display). The f1ori allow the synthesis of a single-stranded DNA and the formation of virions. These virions are produced by E.coli cells that possess these vectors by infecting the cells with helper phage, and nanobodies are easily selected by phage display [75].The technique used for the selection of suitable antibody by the exposing the phage library to an immobilized antigen, named bio-panning [107,108] or panning for short.

Bio-panning

The bio-panning technique is screened for selection antibody fragments or peptides presented on the phage surface are more efficient for clone screening, since it allows isolating clones with defined specificity and affinity [109]. This technique involves the incubation phage-displayed antibodies with an immobilized target antigen [110,111]. Unbound phages are eliminated by washing, while, phages that specifically bind the target are eluted. Specifically bound phage can be eluted from the immobilized antigen using acidic solutions such as "HCl or glycine buffer" [112],

or basic solutions such as triethylamine [113], or this processes can take place by cleavage the protease site incorporated in the recombinant coat protein [114], or occur through competition with excess antigen [110]. In the next step, the eluted phages are amplified in *E. coli.*

Indeed, only one round of selection is required. To avoid the presence of unspecific background phage, several rounds of selection can be repeated and necessary to getting a clone with high affinity to the target antigen (approximately 2-4 rounds).

Production of Antibodies by Phage Display

The top panel a repertoire of scFv is produced with polymerase chain reaction (PCR). The build of repertoires chain can be occur either from VH and Vk genes in vivo or from V gene segment in vitro. The repertoire of a scFv gene is cloned into phage vector in a way that fuses the scFv gene to a gene III that encodes a protein pIII expressed on the phage surface. In the medial panel, the vector directs E.coli to produce phage antibodies, which have a functional scFv fused to pIII on their surface. Inside each phage antibody is the vector DNA containing the gene for the scFv. Phage antibodies binding a specific antigen can be separated from non-binding phage antibodies by affinity on immobilized antigen (bottom panel). A single round of selection increased the number of antigen –binding phage antibodies by a factor ranging from 20 to 1000, depending on antibody affinity. Eluted phage antibodies use infected E.coli which then produces more phage antibodies for the next round of selection. Repeated rounds selection is improve to isolate antigen-binding phage antibodies that were originally found at repeats of less than 1 in a billion [113]. One of the hallmark characteristics of phage display technology is the capacity to produce very large libraries of peptides [93] the power of phage display technology results from its ability to establish a physical connection between phenotype (displayed peptide) and genotype (DNA sequence encoding the displayed peptide). This phenotype-genotype link forms the cornerstone of phage display methodology and makes it possible for researchers to isolate target-avid ligands displayed on the phage.

Vector Systems

The vector also named "plasmid" is extra chromosomal, and double-stranded, generally circular DNA sequences that are capable of automatically replicating in a host and that are found in prokaryotes and eukaryotes [115]. Usually, plasmids often have genes that may support the survival of the organism, for example, antibiotic resistance while the artificial plasmids are commonly used as vectors in molecular cloning, leading to driving the replication of recombinant DNA sequences within the host. In vivo, there are many plasmids, and this study used the one of them is pComb3XSS.

The pComb3XSS plasmid

The pComb3x vector was constructed as anoriginof pComb3x; it's self-derived from the pBluescript vector by Barbas et al., [116]. The Pcomb3XSS system is used for the expression of the Fab fragment, and cloning in E. coli, carried the nanobody gene fragment (obtained from the immunization camel), with phagemid pComb3XSS for a library construction.

The pComb3XSS plasmid has many features are good for cloning:

- The plasmid contains an origin replication that facilitates the semi-independent replication of plasmid within the host .
- The plasmid has genes encoded for antibiotic resistance which helps the host cell to maintenance in an environment.

- Plasmid presents widely in many bacteria, like Escherichia coli, and may also be found in some eukaryotes such as yeast, Saccharomyces cerevisiae [117].
- The bacteria containing the plasmids can produce millions of copies of the vector within in hours, and the amplified vectors can be then extracted for further manipulation.
- The plasmid has multiple restriction enzyme cleavage sites that allow for the insertion of the desired fragment insert which is benefit used for cloning purposes in the laboratory.

Cancer.

In general, the cells in the body divide grow and die in an orderly manner, but sometimes these cells divide abnormally and uncontrolled [118] which leads to call Cancer. Cancer is the global disease leading cause of death [119], which is caused by genetic mutation or abnormal metabolism observed within cells. The factors causing the cancer are:

- External factors like (tobacco, an infectious organism, chemicals, and radiation) [120-122].
- Internal factors like inherited mutation, hormones, immune condition and mutations that occur from metabolism [120,122,123].

There are different types of receptors on some cells of the body are hormone receptors are proteins found on normal some cells that take up the hormone signals telling the cells to grow like estrogen receptor (ER), progesterone receptor (PR) and protein receptors called human epidermal growth factor receptor (HER2). These receptors play a critical role in the growth of normal cells and developing cancer. In this study will focus on protein receptors human epidermal growth factor 2 (HER2).

Epidermal Growth Factor Receptor Family (EGFR)

The epidermal growth factor receptor (EGFR), also named ErbB protein, belongs to a family tyrosine kinase. This family of receptors is expressed in normal tissues and plays in the control of most fundamental cellular processes like the cell cycle, cell migration, cell metabolism and survival, as well as cell proliferation and differentiation, also plays role in the pathogenesis of several human cancers and another disease:

- Insufficient ErbB signaling is associated with the development of neurodegenerative diseases in humans, such as multiple sclerosis and Alzheimer's disease [124].
- Excessive ErbB signaling is associated with the development of a wide variety of types of solid tumor.

The HER family is made up of four main members [125-129] are including:

- HER-1 (ErbB1).
- HER-2 (ErbB2).
- HER-3 (ErbB3).
- HER-4 (ErbB4).

These proteins are encoded by distinct genes that are expressed on chromosomes 7, 17, 12, and 2, respectively.

Proteins in the EGFR family (HER2, HER2, HER3, and HER4) are all trans-membrane proteins sharing a common basic molecular structure, which are composed of three major distinct regions, they are:

- N – Terminal extracellular domain (ECD).
- Single α- helix trans-membrane domain(TM).
- Intracellular tyrosine kinase domain [130].

Human Epidermal Growth Factor Receptor 2 (HER2)

The human epidermal growth factor receptor 2 (HER2) is a glycoprotein with several alternative names like (ErbB2, CD340, or proto-oncogene neu or $p185^{HER2}$) [131,132]. The human epidermal growth factor receptor 2 consists of 1255 amino acid, they live on the outside of some cells and receive signals from the body [133]; its encoded by the HER2/neu oncogenic located at the long arm of human chromosome 17 [134].

Correlation between Human Epidermal Growth Factor Receptor 2 and Cancer.

In normal cells, activation of HER2 controls normal cell growth, differentiation, and motility [135], and the number of HER2 receptors per cell is between 20000-50000,while in cancer cells deregulation of these pathways and increased expression of HER-2/neu to 2 million [136] promotes tumor cell growth and migration. Overexpression of HER2 is a correlation with HER2 gene. Amplification of the HER2 gene and over expression of HER2 protein product induces cell transformation and then lead to tumor aggressiveness and an increased probability for recurrent disease.

The human epidermal growth factor receptor 2 (HER2) is expressed in many tissues and its major role in these tissues is to facilitate excessive/uncontrolled cell growth and tumorigenesis [134,137], and becomes more aggressive and more resistant for treatment For example in the breast cancer, HER2 over-expression about 20% – 30% [27], gastric cancer [138], salivary duct carcinomas [139], esophageal cancer, ovarian cancer, stomach and adenocarcinoma of the lung [140], pancreatic cancer and uterine serous endometrial cancer [141].

Breast cancer is the most common malignancy and the leading cause of cancer-related death among women all around the world [142] and it is after lung cancer [143]. Breast cancer is a complex and heterogeneous disease, molecular profile, and clinical behavior which require different treatment [144]. The gene amplification and/ or over expression of HER2 occur in 20 - 30% of breast cancer [145-147], that's lead to increased cell proliferation, tumor invasiveness, accelerated angiogenesis and reduced apoptosis which ultimately translate into aggressive disease, resistance to traditional systemic therapy and decrease survival [148,149].

Mechanism of Human Epidermal Growth Factor Receptor 2 in Breast Cancer and signaling Pathway

The human epidermal growth factor receptor 2 (HER2) different from other EGFR family members, is as an "orphan receptor "due to lacking a known ligand [150]. HER2 signaling is a complex network comprised of membrane receptor and their ligands protein kinase and regulating genes that affect various cellular functions. The formation HER2 is either heterodimers or homodimer, and after activates the intracellular tyrosine kinase, then triggers the autophosphorylation of specific tyrosine residues. The phosphorylation of tyrosine in turn adaptor proteins or enzymes to initiate a succession of signaling cascades to regulate cellular processes [151,152]. The induction of PI3K signal activities is aroused by the heterodimer composed of HER2 and HER3. However, as for as Ras/Raf/MAPK signaling pathway, it can be activated by all of the dimers containing HER2 "HER1/HER2, HER2/HER2, HER2/HER3 and HER2/HER4" [153]. The HER2 pathway has been described in systems biology terms as a complex biological network comprised of three layers, an input layer of membrane receptors and their ligands to trigger the signal coming from outside the cell, a core system processing layer of protein kinases transmitting the signal to the nucleus, and an output layer of transcription factors regulating genes that affect various cellular functions.

Diagnosis of Positive Human Epidermal Growth Factor Receptor 2 in Breast Cancer.

There are various methods for determining HER2 status, like immunohistochemistry (IHC), enzyme-linked immune-sorbent assay (ELISA) analysis and Western blot test for HER2 protein over expression, while fluorescence in situ hybridization (FISH), chromogenic in situ hybridization (CISH), silver in situ hybridization (SISH), Southern blot and polymerase chain reaction (PCR) for HER2/neu gene amplification [154,155]. HER2 positive of breast cancer remains a threatening health problem in both developed and developing countries a critical target for developing therapeutic drugs against HER2 positive of breast cancer, and there are many therapies for treating of the breast cancer depending on the diagnosis.

Types of cancer therapies

There are four types of cancer therapies:

1. Chemotherapy: is a type of cancer treatment, and involves a large group of cytotoxic drugs that interfere with cell division and DNA synthesis, and then kill cancer cells.

2. Hormonal therapy: is a cancer treatment that slows or stops the growth of cancer that uses hormones to grow. Hormone therapy is additionally known as hormonal therapy, hormone treatment, or endocrine therapy.

3. Targeted therapy: It is a type of cancer treatment that targets the changes in cancer cells that help them grow, divide, and spread, and most targeted therapies are either small-molecule drugs that are a small enough to enter cells easily, so they are used for targets that are inside cell or monoclonal antibodies are drugs that are not able to enter cells easily. Instead, they attach to specific targets on the outer surface of cancer cells

4. Immunotherapy: is a type of treatment that helps the immune system fight cancer. There are several types of immunotherapy used to treat cancer. These treatments can either help the immune system attack cancer directly or stimulate the immune system in a more general way.

Many treatments targeted the HER2 by clinically approved drugs. The two keys domains of the HER2: the extracellular domain and the tyrosine kinase domain have been targeted to block the HER2 signaling. There are many antibodies targeting the extracellular domain of HER2 that exists in an open conformation and is a suitable partner for another receptor for dimerization. The dimerization results in the phosphorylation of the intracellular tyrosine kinase domain and activation of downstream signaling pathways. Monoclonal antibodies (MAbs) targeting the extracellular domain of HER2 can be used to suppress its dimerization with other HER family members and therefore block the pathways [153]. Immunoglobulin therapies, also known as a normal human immunoglobulin (NHIG) and used to treat a number of breast cancers, example is: Trastuzumab, Pertuzumab and Lapatinib.

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