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Production of Recombinant Human EGFR CR2 Domain and Generation of Monoclonal Antibodies Discriminating The R and K Variants.

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Abstract

Background: The epidermal growth factor receptor (EGFR) was the first molecular marker to be targeted successfully in monoclonal antibodies-based cancer immunotherapy. The human EGFR displays two common natural genetic variants (R521K) located in the molecule extracellular CR2 domain. This polymorphism affects the outcome of cancer immunotherapy using anti-EGFR mAbs.

Results: In this paper, we report the production, purification and characterization of recombinant forms of the EGFR/CR2-R and CR2-K variants as Glutathione S-transferase (GST) fusion protein in *E. Coli* BL21. We used these two proteins to generate three different murine monoclonal antibodies to the EGFR CR2 domain (anti-CR2R, anti-CR2K and anti-CR2-RK). We carried out the molecular characterization of the anti-CR2-RK mAb. Analysis using Western blot, ELISA and Immunohistochemistry of various tumor tissues samples, showed that anti-CR2RK mAb was specific of the human EGFR CR2 extracellular domain and recognizes equally both the CR2-K and CR2-R natural genetic variants. In addition, the affinity binding of anti-CR2-RK mAb, as determined by Surface Plasmon Resonance, was equal to 27.7KD µM.

Conclusions: We produced recombinant forms of the human EGFR CR2 domain R and K variants and generated mAbs that discriminate between them. These mAbs can be engineered into novel cancer therapeutic and or diagnostic tools tailored to the patients EGFR genetic makeup.

Background.

The epidermal growth factor receptor (EGFR) is an important member of the four homologous transmembrane proteins that mediate the actions of growth factors like EGF (1). It's a transmembrane receptor consisting of five portions; an extracellular domain, a transmembrane domain, a juxta membrane (JM) segments, an intracellular tyrosine kinase domain and a C-terminal regulatory tail (2). The EGFR plays a crucial role in the ligand binding and the initiation of intracellular signaling pathways which affect the biochemical state of the cell (3). The mechanisms and signaling cascades of EGFR influences processes that are linked to progression of tumor cells such as proliferation, cell survival, angiogenesis, migration, and invasion (4). Therefore, EGFR is involved in many types of human cancers including head and neck cancer, lung, ovarian, breast, bladder, colon, pancreatic and glioblastoma (5). Moreover, some EGFR sequence variations that affect the signaling of the receptor are linked to different kinds of cancers. Indeed, EGFR polymorphism in the coding and regulatory sequences impact the cell surface expression level and/or activity of EGFR (6). Consequently, polymorphic variants in EGFR influence several of its biological activities, like ligands affinity, dimerization efficacy, kinase activity, expression levels, with a consequent impact in signaling pathways and cell behavior (2). Meanwhile, some variants have positive impact on the effectiveness of anticancer agents. For example, the genetic variant of EGFR R521K affects the therapeutic efficiency of the inhibitors used to treat patients suffering from colorectal cancer (7)

Single nucleotide polymorphisms (SNPs) are widespread in genes encoding all of the 4 members of ErbB receptor family. Somatic mutations identified in *ErbB* genes including EGFR influence the receptor activity (8). A major SNP (rs2227983) in EGFR correspond to a substitution of guanine (G) to adenine (A) affecting the residue at position 521. It is located in exon 13 that encodes a part of the extracellular region of *EGFR* and results in the substitution of an arginine (R) with lysine (K). The R521K variant is a widespread functional SNP that plays an important role in EGFR expressing tumors such as gliomas, lung and CRC cancer (9). The study of the R521K polymorphism effect on clinicopathologic features in colorectal carcinoma patients, suggested that this polymorphism might be a key determinant to reduce tumor recurrence in advanced colorectal carcinoma in patients who received curative surgery and a longer survival for patients with advanced and metastatic colorectal carcinoma. This was attributed to the fact that R521K polymorphism has attenuated functions in ligand binding, tyrosine kinase activation and growth stimulation (10). In the last two decades, monoclonal antibodies (mAbs) were approved for a variety of applications because of their specificity and affinity for targeted antigens (11). They were used in several approaches especially as immunotherapeutic tools. Several mAbs were approved by the US Food and Drug Administration (FDA) for the treatment of solid tumors and most of them were developed to recognize targets like HER2, EGFR, VEGF/R, GD2, PD-1 and RANKL (12). Similarly, the rate of product approvals and sales of mAb products in the market value has increased rapidly, with global sales revenue for all mAb products at $115.2 billion in 2018 (13). The high specificity of mAbs for their targets makes them effective therapeutic agents against extracellular domain of EGFR (14) (15). This study focused on cloning, expression, purification of recombinant forms of the wild type and the natural R and K variants of the CR2 ligand-binding domain of the human EGFR. In addition. It reports the generation of mAbs that discriminate between the human

EGFR R and K variants as well as the molecular characterization and cellular recognition pattern of "mAb-EGFR/CR2-RK" that recognizes both variants.

Results.

Cloning of CR2 domain gene into the expression vector

The full-length cDNAs of the wild type CR2 domain and its mutated variant were initially synthesized and cloned into pGEX4T-1 plasmid (Figure 1A & 1B). The CR2-R, CR2-K sequences were flanked by the restriction sites *EcoRI* and *XhoI* (Figure 1C & 1D). When fused to the gene of interest, the GST fusion enables the protein to be purified through single-step GST-affinity column purification. Recombinant expression plasmids (pGEX4T-1-W-CR2-R and pGEX4T-1-W-CR2-K) were efficiently transformed into *E. coli* BL21 strain using electroporation technique. Positive clones were confirmed by colony PCR using specific primers targeting CR2 sequences. The predicted size for the CR2 amplicon was 552 bp (Figure 2A & 2B). The recombinant plasmids were purified, sequenced and the sequences analyzed by nucleotide Blast server (BLAST RefSeqGene) from National Center for Biotechnology Information (NCBI)http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&BLAST_PROGRAMS=megaBlast&PAGE_TYPE=BlastSearch&SHOW_DEFAULTS=on&BLAST_SPEC=RefseqGene.

Expression and purification strategy of recombinant proteins

The expression of the recombinant proteins CR2-R and CR2-K was carried out in *E. coli BL21* (DE3). The host cells were induced at an OD of 0.8 with 0.25 Mm IPTG for 4 hours under agitation (250 rpm). Soluble proteins (supernatants) were resolved on 12% SDS polyacrylamide gel, stained with Coomassie Blue R-250 after estimation of the proteins concentration using Bradford assay. The expected size for the recombinant CR2 fusion protein and its mutant variant were observed at ~46 kDa, whereas the expected size for the free GST bands were observed at ~26kDa (Figure 3A). In addition, the presence of an immunoreactive band of ~46 kDa in western blot analysis using anti-GST antibody confirmed the expression of the proteins (Figure 3B). GST tagged recombinant CR2-R and CR2-K were purified using 3 ml GST purification column. Based on the results, a single band of approximately ~46 kDa on a Coomassie blue stained (SDS-PAGE) demonstrated the presence of highly purified (>90%) proteins (Figure 3C). In addition, a significant amount of unbound GST tagged proteins (flow through) was also observed.

Monoclonal antibodies generation by hybridoma technology

The production of the mAbs was carried out in collaboration with ProMab Biotechnology, Inc (California, U.S.A). After the immunization with a conjugated purified free peptide or the recombinant proteins, ELISA assay was used to determine the highest titer mice to serve for cell fusion. Fusion of immunized mice splenocytes with SP2/0 myeloma cells generated 10 hybridoma clones secreting antibody specific to CR2R and 10 clones secreting antibody specific to CR2K. The supernatants from all the 20 clones were tested by ELISA utilizing purified proteins [CR2R and CR2K] and free peptides as antigens to select the best antibody-producing hybridomas. ELISA results clearly show that all supernatant obtained from CR2K antibody-producing hybridoma clones (n=10) contain antibodies recognizing specifically CR2K and do not cross react with CR2R protein (Figure 4). However, we found that only 5 clones among the CR2R antibody-producing hybridoma generate antibody recognizing specifically CR2R protein: 3C11H5, 3C11D3, 6B7A1, 6B7D2 and 8B5B1 (Figure 5). The last 5 clones (3C9H12, 3C9B8, 4A6B5, 4A6D3 and 5B9A1) cross react with CR2K protein (Figure 5). Therefore, we selected 2 clones “2D7C8 and 5B9C8” secreting monoclonal antibody specific to CR2K (based on the highest OD reading suggesting high affinity) and 2 clones “6B7D2, 3C11D3” producing monoclonal antibody specific to CR2R. We also selected clones 4A6D3 and 5B9A1 that produce monoclonal antibody recognizing both proteins CR2R and CR2K. The selected hybridoma clones were cloned and expanded.

Monoclonal antibody isotype characterization

Isotyping of monoclonal antibody was carried out with mouse monoclonal antibody isotyping test kit from Zymed. The isotype of mAb specific to CR2K (2D7C8 and 5B9C8) is an IgG3 with a kappa (κ) light chain. The isotype for 3C11D3 and 6B7D2 mAb specific to CR2R as well as 4A6D3 and 5B9A1 clones that recognize both proteins, were IgG1 with a kappa (k) light chain (Table 1).

Table 1: Summary of the monoclonal antibodies isotype characterization.

Clone ID	CR2R		CR2K		CR2RK	
Isotype	3C11D3	6B7D2	2D7C8	5B9C8	4A6D3	5B9A1
IgG1	P	P	-	-	P	P
IgG3	-	-	P	P	-	-
κ	P	P	P	P	P	P

Molecular and Cellular Characterization of mAb-EGFR/CR2-RK

Three purified monoclonal antibodies that recognize the EGFR CR2 domain R and K variants were successfully generated. The molecular and cellular characterization of mAb-EGFR/CR2-RK was carried out because of its specificity for both variants of CR2 domain, the wild type and mutated form. Besides, it was isotyped as an IgG1 subclass, which is the most widely, used subtype in cancer therapeutic applications.

A. Western blot analysis

We first determined whether the mAb-EGFR/CR2-RK can specifically recognize purified CR2 recombinant proteins in Western blots. The result showed a specific band at ~46 kDa, confirming that this mAb was specific for the two mutations and would function in Western blots (Figure 6).

B. Enzyme linked immunosorbent assay (ELISA) procedure

ELISA technique was used to test the specificity and sensitivity of mAb-EGFR/CR2-RK against the recombinant CR2R and CR2K antigens. According to the optical density reading of the ELISA assay, the mAb-EGFR/CR2-RK recognizes both CR2R and CR2K antigens with a slightly different sensitivity (Figure 7) and (Figure 8).

C. Immunohistochemistry (IHC) study

We next asked whether this mAb-EGFR/CR2-RK was able to recognize mutant EGFRs in paraffin embedded tissues sections from lung adenocarcinoma of cancer patients in immunohistochemical tests. Apparent EGFR expression of cells surface was seen using VENTANA mAb-EGFR/CR2-RK against human EGFR protein. By applying the scoring system for HER2/neu expression accredited by the college of American pathologist for use in breast cancer diagnosis, a positive reaction to the EGFR antibody (mAb-EGFR/CR2-RK) was considered when a consistent brown color appeared on the cell membranous area. Representative examples for different scores are shown in Figure 9A-9C. Furthermore, normal skin tissue was stained in the absence of primary antibody as a negative control (Figure 9D). Therefore, Western blotting and immunohistochemical analysis consistently showed that mAb-EGFR/CR2-RK was able to identify the appropriate EGFR-CR2 mutations.

D. Affinity Measurement.

We used Biacore to measure the direct binding interactions between mAb-EGFR/CR2RK and the two CR2R and CR2K recombinant antigens. Mab-EGFR/CR2RK was used as ligand and the antigens as analytes (Figure 10). The kinetic parameters measured are the following: Association constant, Ka=0.6059X10^{6}M^{-1} S^{-1}; dissociation constant, Kd= 0.01678 S^{-1} and equilibrium dissociation constant, KD= 27.7 μM. The data showed a binding affinity of 27.7KD μM for mAb EGFR/CR2RK.

Discussion.

The EGFR is involved in various cellular functions through cell growth, differentiation, migration, apoptosis and survival for over 70% of all cancer cells (16). The overexpression of EGFR ligands, amplification of EGFR, and the prolonged activation in mutated EGFR-TK may change the intracellular signaling pathways of the cells. Accordingly, EGFR is the major target for prophylactic and therapeutic intervention in various kinds of cancer. In recent years, several agents have shown efficacy in targeting EGFR such as peptides, nanobodies and affibodies (17) (18) (19). These days, concerted efforts are being focused to recognize and identify molecular markers that can predict patients who are more likely to respond to anti-EGFR therapy efficiently; and to search for combinational methods with EGFR inhibitors and try to discover new therapeutic agents with better clinical effectiveness (20). The

two major approaches that succeeded in clinical trials for targeting EGFR were monoclonal antibodies and tyrosine kinase inhibitors (21). These two strategies have shown activity in many types of advanced human cancer and their role in the treatment of earlier tumors stages is presently being evaluated (22). Moreover, they have entered clinical phase III trials and were approved as first line indication in countries like US and several European states (23).

A previous mutational study that we carried out (24) revealed that 48% of Arabian cancer patients and 47.36% of healthy people exhibit the G→A transition, resulting in the amino acid substitution of arginine by lysine in position 521 [R521K]. This SNP is located at the boundary between EGFR domain III (the direct interaction site with cetuximab) and domain IV. In addition many studies showed its importance and significance to make the cell more sensitive to targeted receptor inhibition through a specific monoclonal antibody (25). In this work, we generated monoclonal antibodies that target the R521K polymorphism and can eventually discriminate between the two variants. First, we constructed 2 prokaryotic expression plasmid CR2-R-pGEX4T-1 and CR2-K-pGEX4T-1, cloned, expressed and purified recombinant wild type (CR2R) and the most common mutated variant of the CR2 sub domain (CR2K) of human EGFR receptor to produce an anti-CR2-R and anti-CR2-K monoclonal antibodies. The genes corresponding to the CR2 domain variants were synthesized and cloned into pGEX4T -1 plasmid. We used *E.coli* BL21 strain because of the suitability of this strain to support the expression of good quality recombinant protein (26). The Expression of both genes was carried out in BL21-λDE3 at 37˚C. Significant amount of soluble protein was found in soluble form for CR2-R and CR2-K as observed in SDS-PAGE and Western blotting. Following Purification, and endotoxin removal, the recombinant CR2-R and CR2-K proteins were used to immunize mice and generate specific mAbs.

As noted, we choose R521K polymorphic variants to be the basic target for our new recombinant monoclonal antibodies. Out of the 3 different mouse mAbs we generated, two have very narrow specificity either to the R or K variant. These two antibodies are to our best knowledge the first mAbs to distinguish between the two natural variants of the human EGFR natural's variants. These antibodies were isotyped as IgG1 (CR2-R) and IgG3 (CR2-K). The third antibody (CR2-RK) recognizes the two variants of EGFR CR2 domain, and likely a different epitope. This mAb was isotyped as IgG1. It is reported that for the selection of the best IgG subclasses in developing therapeutic monoclonal antibody drugs several criteria such as stability, flexibility, mediation of antibody dependent cell cytotoxicity (ADCC), mediation of cellular dependent cytotoxicity (CDC), and C1q protein binding (27) need to be considered. Indeed, IgG1 subclass is one of the most used in antibody therapy because of its stability, half-life, less aggregation formation, higher serum concentration. In another hand, it is established that the Fc-FcγR interaction of a given IgG Fc plays the same important role as the Fab-antigen interaction for anti-tumor antibodies. Therefore, to design single targeted therapeutic antibodies as anti-tumor drugs, significant aspects must be carefully considered like the biological characteristics of the targets, the corresponding cells expressing the targets, and the mechanism of actions proposed for the therapeutic antibodies. IgG1 mAbs are the first choice because of their high affinity for all FcγRs and their capacity to induce ADCC and ADCP (28). Indeed, the majority of mAbs approved by the Food and Drug Administration (FDA) and the European Medicines Agency (EMA) and currently marketed, as anti-cancer drugs are predominantly IgG1. Most of these mAbs activate both the humoral and cellular immune system for the immunological control of tumor growth and metastasis (29). Whereas, IgG3 sub class was not used in cancer therapy because of the long hinge region leading to high chances of aggregation and instability, short half-life and its extensive allotypic polymorphism (30).

Therefore, in this study we focused on the characterization of the molecular features and cellular recognition pattern of mAb "EGFR/CR2RK". Our aim was to develop an advanced version "Biobetter" of the anti-human EGFR mAbs currently available in the market such as "Cetuximab" that has proven to be very successful in the treatment of malignancies such as colorectal and head and neck carcinoma as well as glioma. The development of murine "mAb-EGFR/CR2-RK" was the first phase toward this end and this study represents the first phase of determining the features of this original anti human EGFR mAb.

The two most important features therapeutic mAbs are affinity and specificity (31). Affinity measures the binding strength between an epitope to an individual paratope (antigen-binding site) on the antibody. High affinity antibodies bind rapidly to the antigen, allowing for greater sensitivity in assays and maintain this bond more readily under inappropriate conditions. At the opposite, low affinity antibodies, bind weakly to the antigen and often do not detect the antigen *in-vivo* or in *in-vitro* binding assays. The affinity of "mAb-EGFR/CR2RK" was determined using label free Surface Plasmon Resonance (SPR)-based biosensors, on a Biacore technical platform. Indeed, SPR is a valuable tool for evaluating therapeutic antibodies with different cases of cancer. SPR was used to compare the immunogenicity of a set of genetically engineered variants of a potential therapeutic antibody against a

protein expressed by a variety of carcinomas (32). In this study, the antigen that is the EGFR/CR2 domain was from recombinant source. Indeed, recombinant antigens are very useful since many steps in mAb development process rely on the use of good amount of purified antigens. Therefore, in the SPR/Biacore analysis the two recombinant genetic variants of the EGFR/CR2 domain were used as analytes while "mAb-EGFR/CR2RK" the ligand, was immobilized on the chip. The association constant (ka), the dissociation constant (kd) and the equilibrium dissociation kinetic parameters KD obtained were suboptimal. Indeed, the antibodies usually have very tight binding to protein antigens and KDs are generally in the nano or picomolar ranges. This is not the case for "mAbEGFR/CR2RK" whose KD is at the micromolar range [27.7 (μM)]. While SPR-based measurements often yield affinities that agree well with solution methods, the use of a surface can sometimes give misleading results due to the introduction of artifacts, which may be influenced by the choice of sensor chip type used and the relative charges of the analyte/ligand pair being studied. However, at this stage of mAb development, the affinity is not really an issue and affinity maturation will be carried out at a later stage.

For the specificity, the two forms of the CR2 antigen were used to show that "mAb-EGFR/CR2-RK" recognizes the EGFR/CR2 domain of both common human EGFR genetic variants R and K at position 521. Nevertheless, in ELISA analysis "mAb-EGFR/CR2-RK" showed a slightly different sensitivity to the R and K variants while clearly recognizing both. This is probably because recombinant proteins from heterologous sources, in our case *E. Coli*, might yield recombinant protein structure that display epitopes that are not quite identical to native epitopes. Therefore, it is of paramount importance for the therapeutic mAb to recognize native epitopes and a mAb cannot be a therapeutic candidate if it does not fulfill this criterion. Thus, the immune histochemical studies were carried out and clearly showed that "mAb-EGFR/CR2-RK" reveals specifically EGFR cell surface expression on a variety of normal and malignant cells. Thus, it can be used to determine the level of EGFR expression and useful as such for tumor diagnosis and grading. The molecular determination of the molecular attributes and the recognition pattern of "mAbEGFR/CR2RK" is a prerequisite in the improvement of a therapeutic mAb for use in human cancer immunotherapy. The data collected from this study showed that "mAbEGFR/CR2RK is eligible for moving to the next step of the development process. In this next step we shall carry out molecular engineering of these antibodies to humanize their sequences, ameliorate their kinetic features through affinity maturation, and further adapt them for personalized treatment particularly for their selective binding to specific variants of the Fcγ receptors CD16 and CD32 in order to achieve optimal personalized clinical efficiency (33).

Conclusions.

We have developed, what is to our best knowledge, the first monoclonal antibodies that discriminate between the EGFR R and K variants. These antibodies can be used to develop novel improved therapeutic tools for cancer based on the EGFR genetic make-up of the patient. In addition, we have completed the characterization of the molecular and the cellular recognition features of 'mAb EGFR/CR2RK" and showed that this murine original monoclonal antibody specific of the human EGFR receptor CR2 extracellular domain, is eligible for the development of personalized antibody-based anti-cancer therapy.

Methods.

Bacterial Strain and plasmid

Escherichia coli *(E. coli)* BL21 was used as host strain with an IPTG-inducible T7 RNA polymerase. And pGEX-4T-1 plasmid (Amersham, Italy) was used for protein construction and maintenance. Recombinant proteins are expressed as fusion protein with Glutathione S transfer (GST). The vector allows high level Isopropyl β-D-1-thiogalactopyranoside (IPTG)-inducible expression for the gene of interest in *E. coli*.

Plasmid Construction

The DNA sequence for the wild type CR2-R domain and its mutant CR2-K domain of human EGFR was obtained from the National Center for Biotechnology Information (NCBI) at https://www.ncbi.nlm.nih.gov/ [Gene ID: 1956]. The wild type of DNA sequences for CR2 domain and its mutated form were flanked with restriction site *EcoRI* and *XhoI*. After that, the wild type of CR2 domain and its mutant form were synthesized and cloned in pGEX4T-1 plasmid (GeneCust, Luxembourg). The resulting construct, named [CR2-R-pGEX4T-1 and CR2-K-pGEX4T-1] were analyzed by DNA sequencing to ascertain its correctness.

Transformation of recombinant plasmids to E. coli BL21

40 ng of the recombinant plasmids (CR2-R-pGEX4T-1 and CR2-K-pGEX4T-1) were transformed into the competent cells of *E. coli* BL21 strain by electroporation using a Gene Pulser Xcell system (Bio-Rad, USA) under the following setting: 200 Ω, 2500 V, 20 µF. Then, 100 µl of the electro-transformed cells were plated onto LB agar medium supplemented with ampicillin at 100 µg/ml as a selective antibiotic. After incubation over night at 37°C; the positive colonies which has recombinant plasmids were picked up and confirmed by colony PCR technique using specific primers targeting CR2 domain (Forward: 5-GAATTCGTGATAATTTCAGGAAAC-3) & (Reverse: 5-CTCGAGCCCATTCGTTGGACAGCC -3). After that, the recombinant plasmids were purified using QIAprep Spin Miniprep Kit (cat# 27106), and were checked by using 1% of agarose gel electrophoresis and were sequenced using a common forward primer for pGEX4T-1 (5-GGGCTGGCAAGCCACGTTTGGTG- 3) and analyzed with the standard nucleotide blast program from NCBI databank.

Recombinant expression of CR2-R and CR2-K proteins in E. coli BL21

The expression of soluble fusion protein for CR2 domain of human EGFR, BL21-λDE3 cells carrying pGEX4T1-CR2-R plasmids, pGEX4T1-CR2-K plasmids and pGEX4T1 empty plasmids were grown at 37˚C at 250 rpm in 3 ml LB media with 100µg/ml Amp to get the OD 600=0.8. Secondary culture was inoculated with 1% inoculums in 20 ml LB culture media having 100µg/ml Amp. Culture was grown till OD 600=0.8 and induced with 0.25 mM IPTG, 37˚C at 250 rpm for 4 hrs. As a control uninduced BL21-λDE3 cells were taken. After cell lysis, the bacterial cells lysate was harvested by centrifugation at 4,000 rpm for 20 min at 4˚C. The pellets were resuspended with 5 ml of lysis buffer in (1/5th of the original culture volume) at 4˚C on ice. The cells were lysed by sonication 6 X 30 seconds (3 minute) with break of 20 seconds (Amplitude 10) on ice. Afterwards, Triton X-100 was added to 1% of lysis volume and mixed on a rocker for 1 hour at 4˚C. finally, the samples were centrifuged at 13200 rpm for 30 minutes at 4˚C and the supernatant was saved at -80˚C considering to be our specific total soluble protein for CR2 domain of human EGFR. The expression was assessed by 15% SDS-PAGE and the protein bands were visualized by staining with Brilliant Blue R-250, followed by Western blotting with anti-GST antibody. Also, the protein concentration was determined using the Bradford method (34).

Western blot analysis

The equivalent amounts of protein samples were resolved on a 15% SDS-PAGE and the separated bands were transferred to polyvinylidene difluoride (PVDF) membrane. The membrane was blocked with 0.1% of 0% fat milk, in Tris-buffered saline, pH 7.4 (TBS) containing 0.05% Tween 20 for 1 hour at room temperature. Western blotting analysis was performed with horseradish peroxidase (HRP)-conjugated anti-GST antibody (Invitrogen) (diluted 1:10000). Target proteins were visualized using an enhanced chemiluminescence (ECL) detection system (Amersham, GE Healthcare).

Purification and endotoxin removal of recombinant protein of CR2 domain

The recombinant proteins CR2-R and CR2-K were applied in GSTrap FF affinity column (GE health, United Kingdom) for purification using the standard procedure described according to manufacture s instruction. The purified proteins (CR2-R and CR2-K) in 1X PBS were passed through Ion Exchange column at PH 7.4 and found in flow through. The flow through were dialysed with 1X PBS under endotoxin free condition at 4˚C. Purified proteins were analyzed on 12% SDS PAGE.

Production of monoclonal antibodies against CR2 domain

The purified proteins (CR2R and CR2-K) were sent to ProMab Biotechnologies, Inc. (California, U.S.A) for custom monoclonal antibodies production. All immunizations and subsequent steps in production of hybridoma cells/purified antibodies were completed by ProMab Biotechnologies, Inc. Briefly, for immunization of Balb/c mice, ProMab Biotechnologies used the recombinant protein as well as the following conjugated free peptides (CWGPEP**R**D and CWGPED**K**D) corresponding to each variant. The antibody response titer was evaluated using ELISA before proceeding to fusion. Approximately 3-4 mg protein immunogen or 2 mg conjugated peptide immunogen and 0.5 mg free were required. Hybridoma fusion was performed using splenocytes from mouse with the best titer and Sp2/0 myeloma cells. After that, supernatants from the growing hybridoma wells were screened by ELISA. 20 clones were positive and sent to life science department in the Arabian Gulf University for evaluation. Two ELISAs assay were performed one using the peptides and the second the recombinant proteins. Six double positive clones

were selected (2 clones specific to CR2R, 2 clones specific to CR2K and two recognizing both CR2R & CR2K,. The monoclonal antibodies isotyping was carried out using Mouse MAb ID kit (HRP): ZYMED Cat#: 90-6550; Lot.60907259.

Reactivity Assessment of the mAbs against hybridomas by ELISA

The reactivity and speci□city of the recombinant monoclonal antibodies examined by ELISA, using hybridoma clones. The free peptide antigens (CWGPEP**R**D) were coated on ELISA plates overnight at 4°C. After being washed with PBS and blocked by 1% BSA, the puri□ed clone supernatant from 24-well plate (primary antibody) was added and incubated for 1 hour at room temperature. The wells were then washed and detected by 3, 3', 5, 5'-Tetramethylbenzidine (TMB) substrate after incubation with HRP labeled anti IgG (secondary antibody). The reaction was stopped by addition of 2M H_2SO_4 and the absorbance was measured at 450 nm by a microtiter ELISA plate reader. The same protocol used with the free peptide antigen (CWGPEP**K**D).

Molecular and Cellular Characterization of mAb EGFR/CR2-RK

We selected 3 purified monoclonal antibodies named mAb-CR2-R (specific for wild type form of CR2 domain), mAb-CR2-K (specific for mutated form of CR2 domain) and mAb-CR2-RK. We carried out molecular and cellular characterization on mAb-CR2-RK it targets both the wild type and mutated form of the CR2 domain.

A. Analysis by western blot

Recombinant CR2 proteins were labelled using the purified anti-CR2RK antibodies in a concentration of 1:10000 in TBST overnight at 4°C with shaking. The membrane was washed as previous and a secondary antibody (anti mouse IgG HRP) diluted in TBS-T (1:4000) was added. The blocking by 0.1% of 0% fat milk for 1 hr. The detection by Amersham ECL Plus western blotting detection kit and LI-COR C-DiGit Chemiluminescence Western Blot Scanner.

B. Analysis by ELISA

Same previous ELISA procedure was used to evaluate the specificity and sensitivity between mAb-EGFR/CR2-RK (50µg/ml) with different concentrations (0.25 mg, 0.5 mg & 1 mg) of both recombinant purified CR2R and CR2K antigens and mAb-EGFR/CR2-RK antibody was tested against each antigen (CR2R & CR2K) in triplicate.

C. Analysis by IHC

The IHC study on formalin-fixed paraffin embedded tissues sections on a total of 10 tissue samples from lung adenocarcinoma of cancer patients from the Arabian patients was carried out at Johns Hopkins Aramco healthcare center. The biopsies were taken before the patients undergo any therapeutic course. The Ventana iView TM DAB detection kit (Cat #760-091) was used to assess the binding of anti-EGFR mAb-CR2-RK with a HRP conjugated anti-mouse IgG as secondary antibody. A normal skin tissue was used as positive tissue control, whereas the same tumor tissue lacking mAb were used as negative control. All the steps of IHC procedure including interpretation of the results, the patient's morphologic findings and pertinent clinical data were confirmed by a qualified pathologist according to the American Society of Clinical Oncology/College of American Pathologists reporting guidelines (ASCO/CAP) using the same standardized reading recommendations of the CAP for Her2neu (35). The intensity of staining was scored using the following scale: no staining, 0; weak staining, 1+; moderate membranous staining in 10-30% of the tumor cells, 2+; and strong staining, 3+ in >30% of tumor cells. We classified scores of 0 and +1 as negative and scores of 2+ and 3+ as positive.

D. Analysis by Surface Plasmon Resonance (Biacore)

The binding affinities between the mAbEGFR/CR2-RK & CR2 recombinant protein was performed using Biacore. The experiment was done at the Biacore Molecular Interactions Shared Resource of Lombardi Comprehensive Cancer Center at Georgetown University in Washington DC, USA. BiacoreT200 (GE Healthcare) was used to determine the kinetic parameters for the binding of Anti-CR2RK-mAb used as a ligand (~150 kDa, 1.5 mg/ml, stock in PBS) with Peptide 1 (labelled as "P1", CWGPEPRD, 959.04kDa, 10 mg/ml stock in PBS) and Peptide 2 (labelled as "P2", CWGPEPKD, 1931.03kDa, 10 mg/ml stock in PBS) were used as analytes. Recombinant GST (~26 kDa, 0.2 mg/ml stock) was also used as a control analyte. The experiments were performed using carboxymethyl-dextran (CM5) sensor chips. Briefly, Flow cell FC1 was used as reference for FC2. Anti-EGFR/CR2-Ab was diluted (1:50 dilution, 30 µg/ml diluted concentration) in HBS-P and captured onto FC2 to a level of ~8000 RU. Right after antibodies were

captured, 20s pulse of NHS-EDC followed by 20s pulse of Ethanolamine were injected in order to crosslink the antibodies onto the surface. Capture/crosslinking was carried out in the presence of HBS-P. Overnight kinetics were performed for the analytes in the presence of the HBS-P. The flow rate of all the solutions was maintained at 50 µL/min. Analyte's concentrations were 0 µM, 62.5 nM, 125 nM, 250 nM, 500 nM, and 1000 nM. One 30s pulse of 1M NaCl was utilized to regenerate sensor surface. Sensorgrams from overnight kinetics were evaluated by 1:1 kinetics model fitting.

Abbreviations

EGFR
Epidermal growth factor receptor.
ELISA
Enzyme-Linked Immunosorbent Assay
mAb
monoclonal antibody
CR1
Cysteine-rich domain 1
CR2
Cysteine-rich domain 2
SNPs
Single nucleotide polymorphisms
CRC
Colorectal cancer.
PCR
Polymerase chain reaction.
E. coli BL21
Escherichia coli.
EGFR-TK
Epidermal growth factor receptor tyrosine kinase
TKIs
Tyrosine kinase inhibitors.
SDS-PAGE
Sodium Dodecyl Sulphate–Polyacrylamide Gel Electrophoresis
ADCC
Antibody-Dependent Cellular Cytotoxicity

Declarations

Ethics approval and consent to participate

The study was reviewed and approved by the Arabian Gulf university research and ethics committee. This article does not contain any studies with human participants or animals performed by any of the authors. Animal experimentation to obtain the 3 mAbs against the recombinant forms of the EGFR/CR2-R and CR2-K variants were entrusted to the ProMab Biotechnologies, Inc. (California, USA, https://www.promab.com/) that use animals under the guidelines of the association for assessment and accreditation of laboratory animal care international (AAALAC) on the protection of animals used for scientific purposes.

Consent for publication

Not applicable.

Availability of data and materials

The data and the materials produced are available from the corresponding.

Competing interests

The authors declare that they have no competing interests.

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Author contributions

M.D.F. conceived the study, designed and interpreted the experiments and secured the funding. S.B-H. overseen all the experimental work and the quality control; M.M. and A.A. carried out all the experiments; W.R. performed IHC technique and analyzed the data, M.M. and A.A. drafted the manuscript. M.D.F. and S.B-H. revised/edited the manuscript. M.M., A.A., S.B-H., W.R. and D.A. were involved in data analysis. All authors read and approved the final manuscript.

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Figures

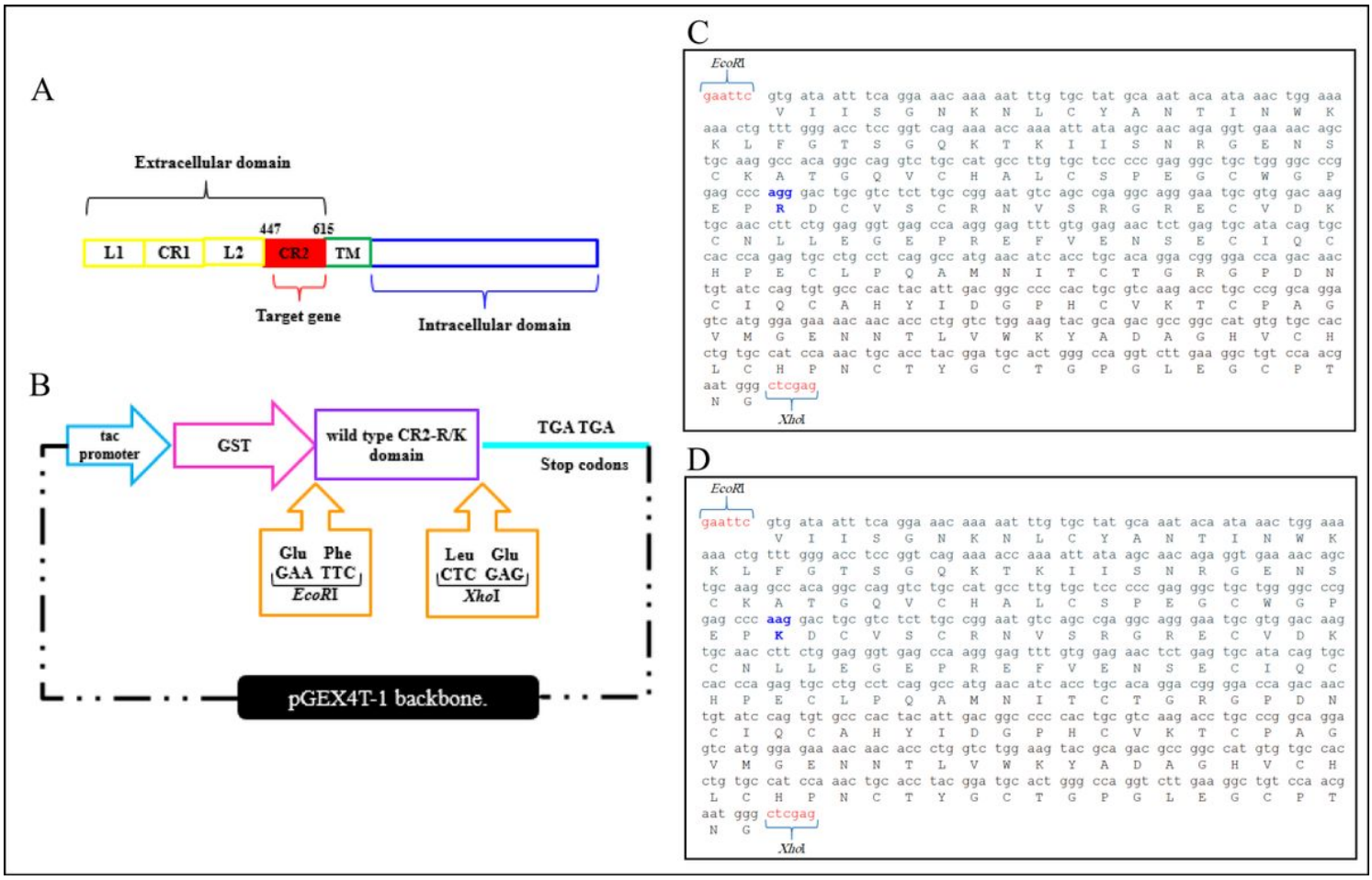

Figure 1

Structure of human EGFR gene and the construction of the recombinant expression plasmid. (A) Schematic representation of domains of the epidermal growth factor receptor. (B) Structure of recombinant plasmid pGEX4T-1 with the target gene wild type CR2 domain (CR2-R) and its mutated variant (CR2-K). (C) Nucleotide and amino acid sequence of wild type CR2-R domain and sequence in blue color is site of mutation. (D) Nucleotide and amino acid sequence of wild type CR2-K domain and sequence in blue color is site of mutation.

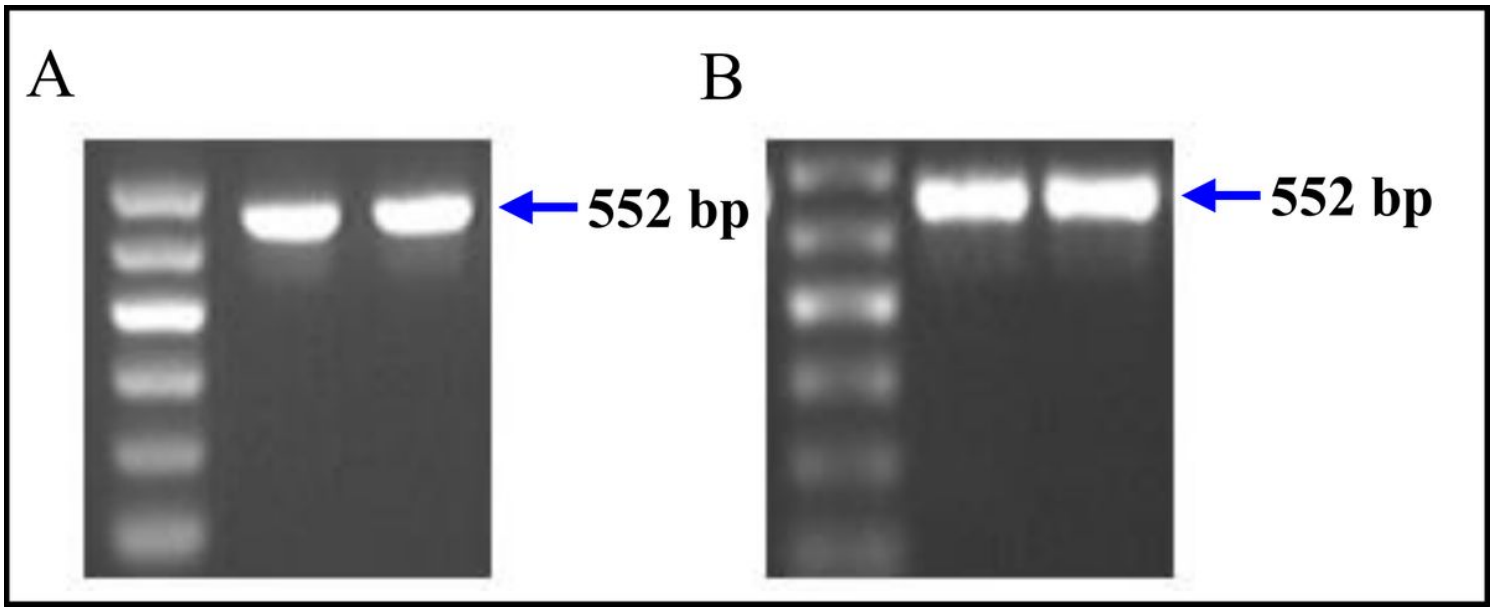

Figure 2

Colony PCR assay results. Agarose gel electrophoresis showing positive wild type CR2-pGEX4T-1 transformants. (A) lane 1 show 100 bp DNA ladder, lane 2-3 show the results for wild type CR2-R-pGEX4T-1 clones (PCR product size=552 bp). (B) lane 1 show 100 bp DNA ladder, lane 2-3 show the results for wild type CR2-K-pGEX4T-1 clones (PCR product size=552 bp).

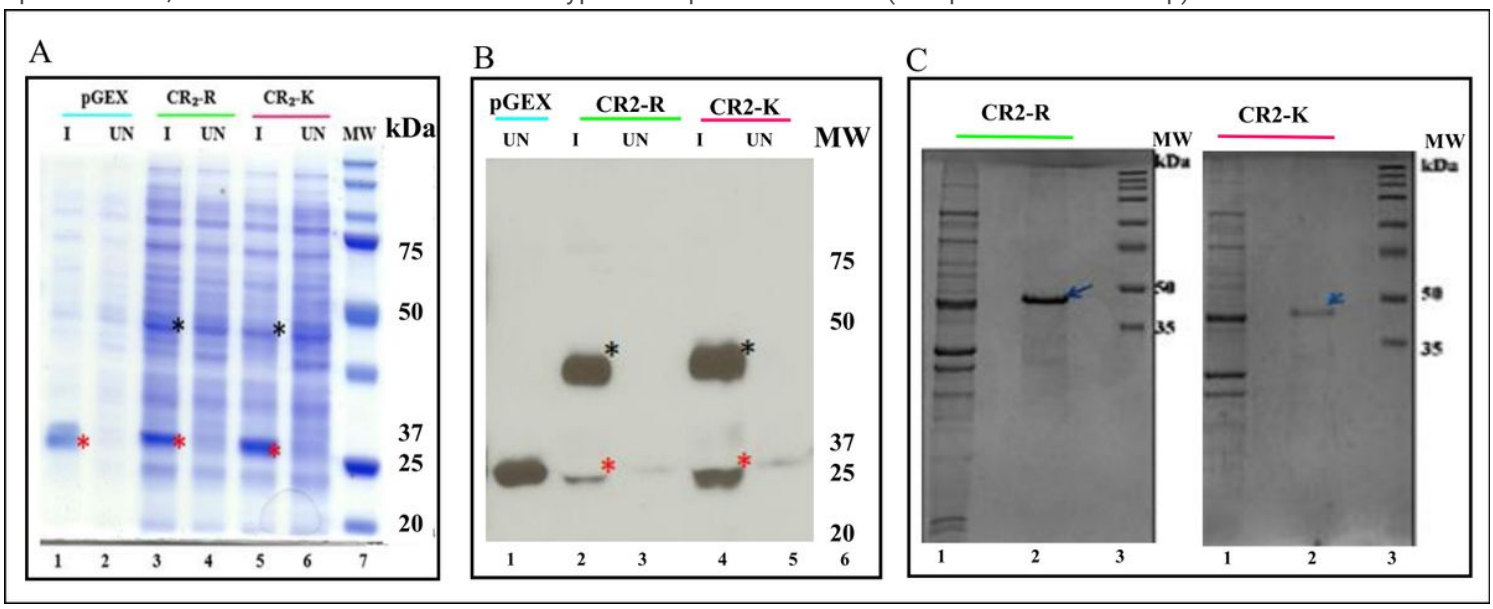

Figure 3

(A) SDS-PAGE analysis of the expression of recombinant proteins CR2-K, CR2-R and pGEX4T-1 as control at 37˚C in BL21 (DE3) cells. The black asterisk indicates the expected size for the target fusion protein (~46 kDa), whereas the red asterisk indicates the free GST bands (~26kDa). I: induced; UN: uninduced culture; MW: molecular weight marker. (B) Recombinant proteins CR2-R and CR2-K identification by immunoblotting using anti-GST antibody. pGEX4T-1: control for protein expression. I: induced; UN: uninduced culture; MW: molecular weight markers. The black asterisks show a band of ~46 kDa corresponding the target fusion protein and the red asterisks indicate a band of ~26 kDa corresponding to free-GST. (C) Purification of CR2-R and CR2-K fusion proteins using GST purification column analyzed by 12% SDS-PAGE. The blue arrows mark the position of the purified fusion proteins for CR2-R and CR2-K (~46 kDa).

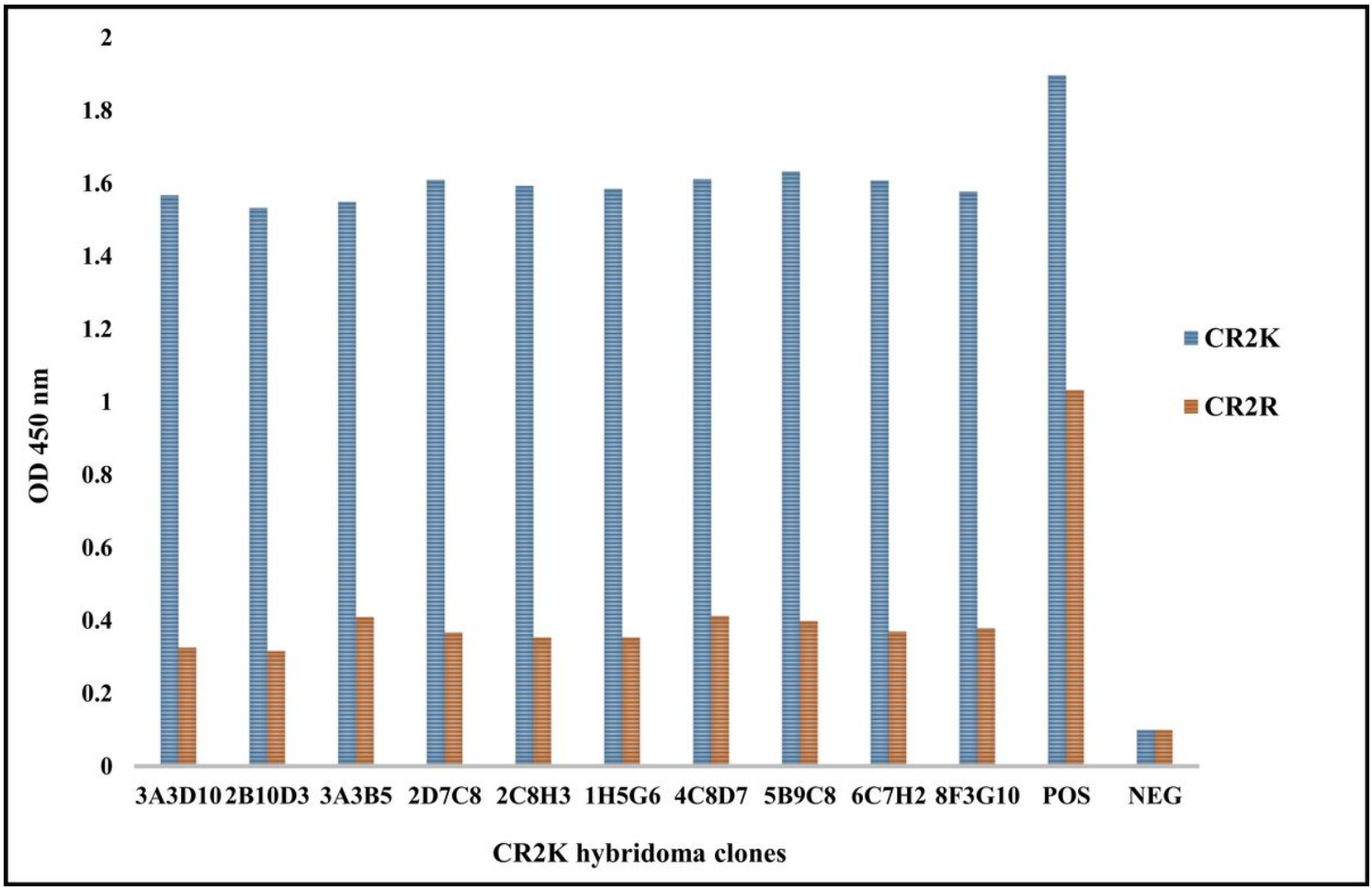

Figure 4

Optical density (OD) reading of ELISA assay for CR2K antibody-producing hybridoma clones. Antigen1: CR2K free peptide (CWGPEPKD); Antigen2: CR2R protein; POS: Positive control is mice immunized anti-serum; NEG: Negative control is mice pre-immune serum.

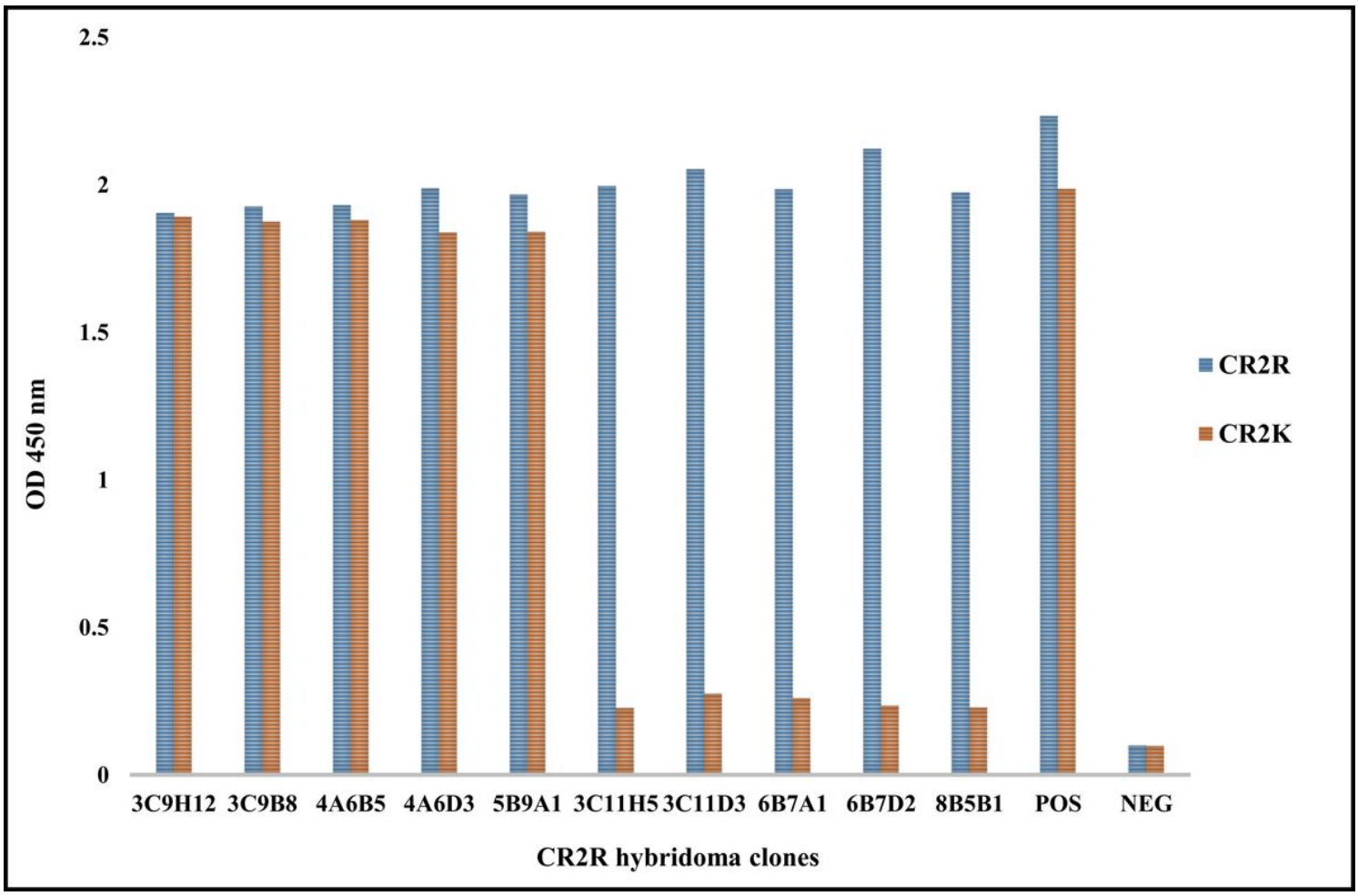

Figure 5

Optical density (OD) reading of ELISA assay for CR2R antibody-producing hybridoma clones. Antigen1: CR2R free peptide (CWGPEPRD); Antigen2: CR2K protein; POS: Positive control is mice immunized anti serum; NEG: Negative control is mice pre-immune serum.

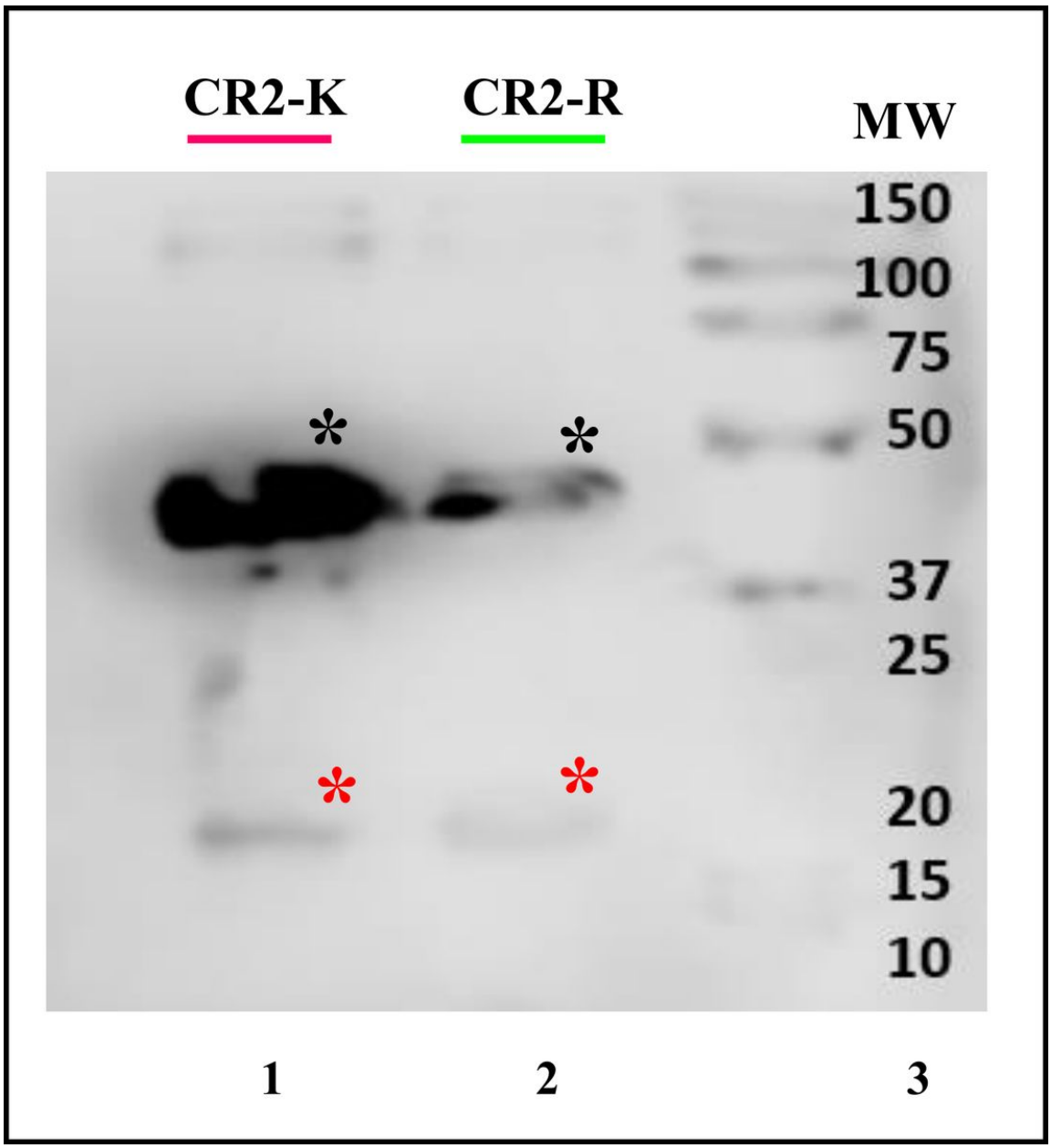

Figure 6

Recombinant proteins CR2-R and CR2-K identification by immunoblotting using anti-GST antibody. The black asterisks show a band of ~46 kDa corresponding the target fusion protein and the red asterisks indicate a band of ~26 kDa corresponding to free-GST. MW: molecular weight markers.

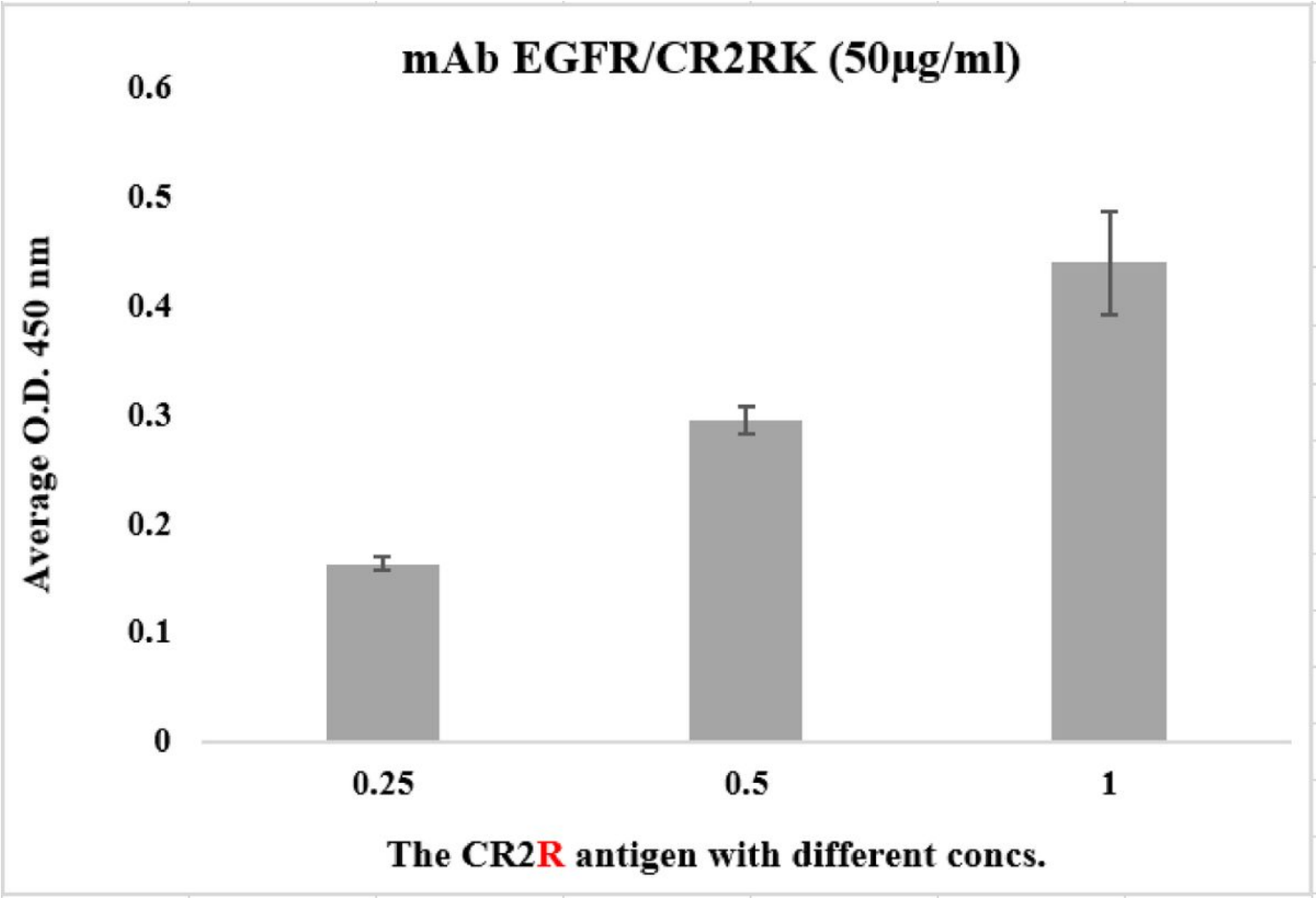

Figure 7

Optical density (OD) reading of ELISA assay for anti mAb-EGFR/CR2RK (50µg/ml) with different concentrations (0.25 mg, 0.5 mg & 1 mg) of CR2R antigen. All experiments were run in triplicates. Error bars are represented in standard deviations.

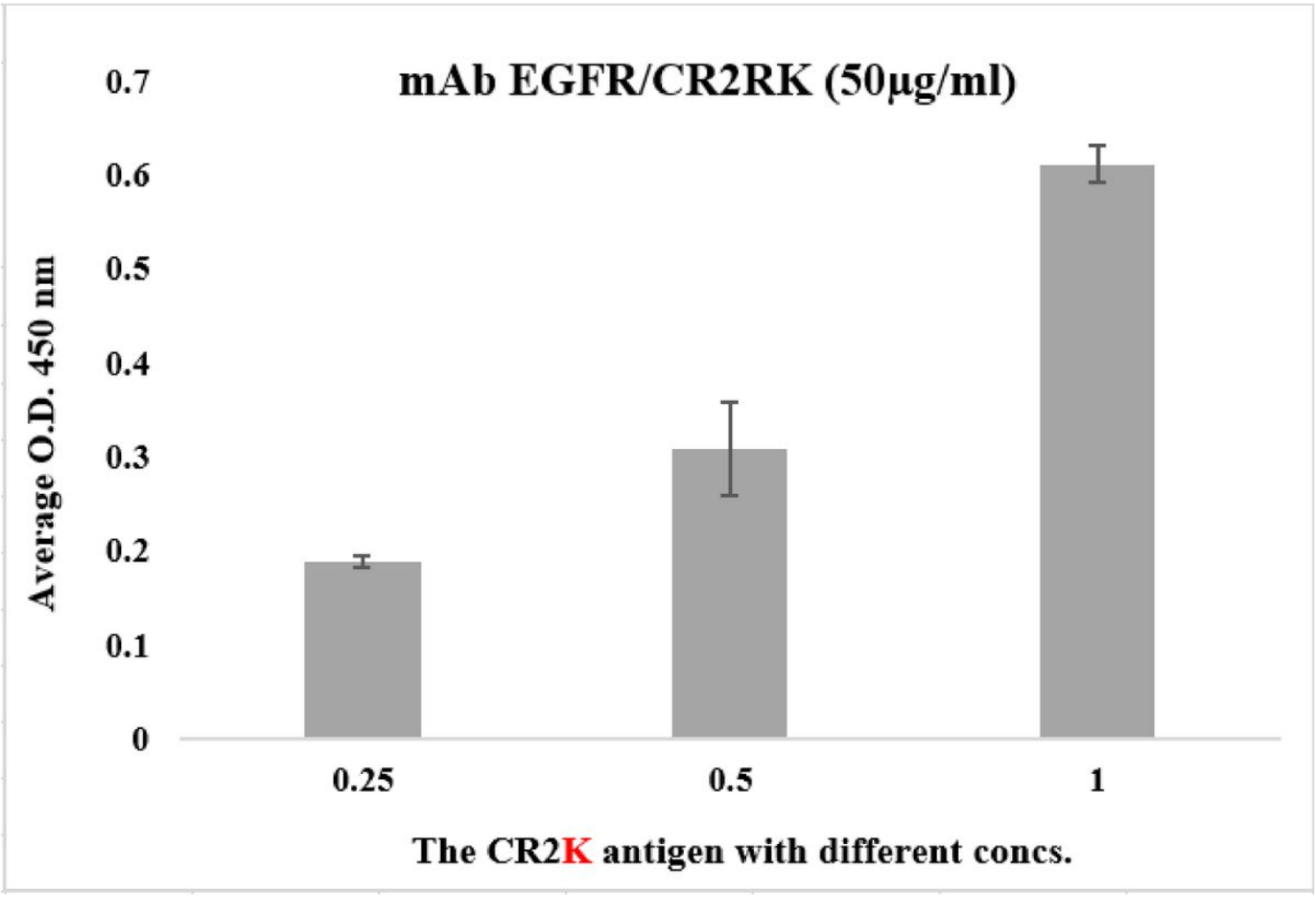

Figure 8

Optical density (OD) reading of ELISA assay for anti mAb-EGFR/CR2RK (50 µg/ml) with different concentrations (0.25 mg, 0.5 mg & 1 mg) of CR2K antigen. All experiments were run in triplicates. Error bars are represented in standard deviations.

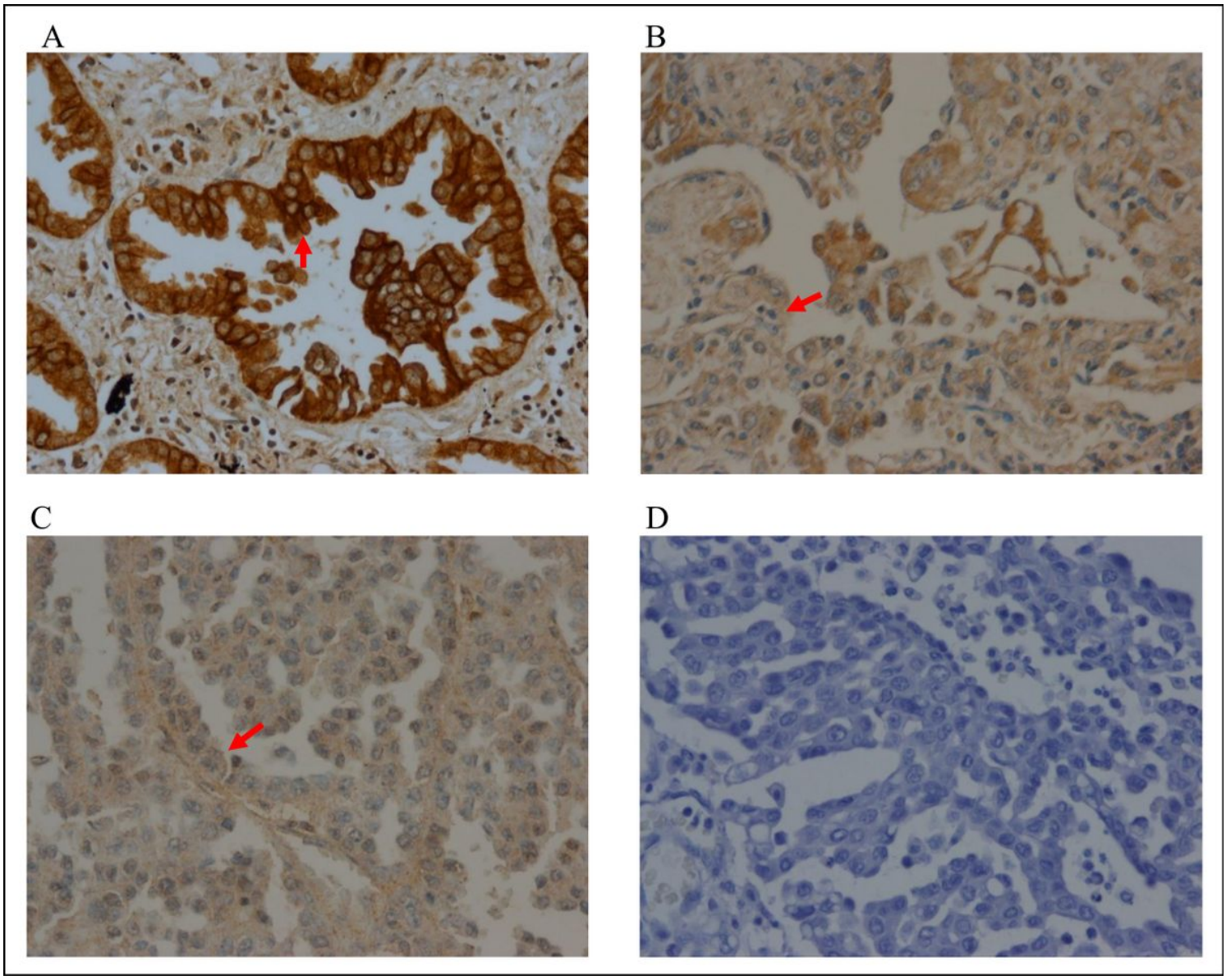

Figure 9

Immunohistochemical staining results for Mab EGFR/CR2RK. The tissue samples from lung adenocarcinoma (LA). The staining of tumor cell membranes with the Ventana Mab EGFR/CR2RK was graded as negative or positive based on the CAP approved standardized scoring (0 - 3+). (A) Shows scored 3+ when >30% of tumor cells exhibiting strong complete membranous immune-reactivity. (B) Shows scored 2+ when weak complete membranous staining in 10-30% of tumor cells. (C) Shows scored 1+ when faint incomplete membranous staining in >10% of tumor cells. (D) Shows scored 0 when negative or non-specific staining. Negative control was obtained by staining in the absence of the primary antibody. Arrow heads in all figures indicate the EGFR membranous brown staining (400 X magnification for all tissue samples).

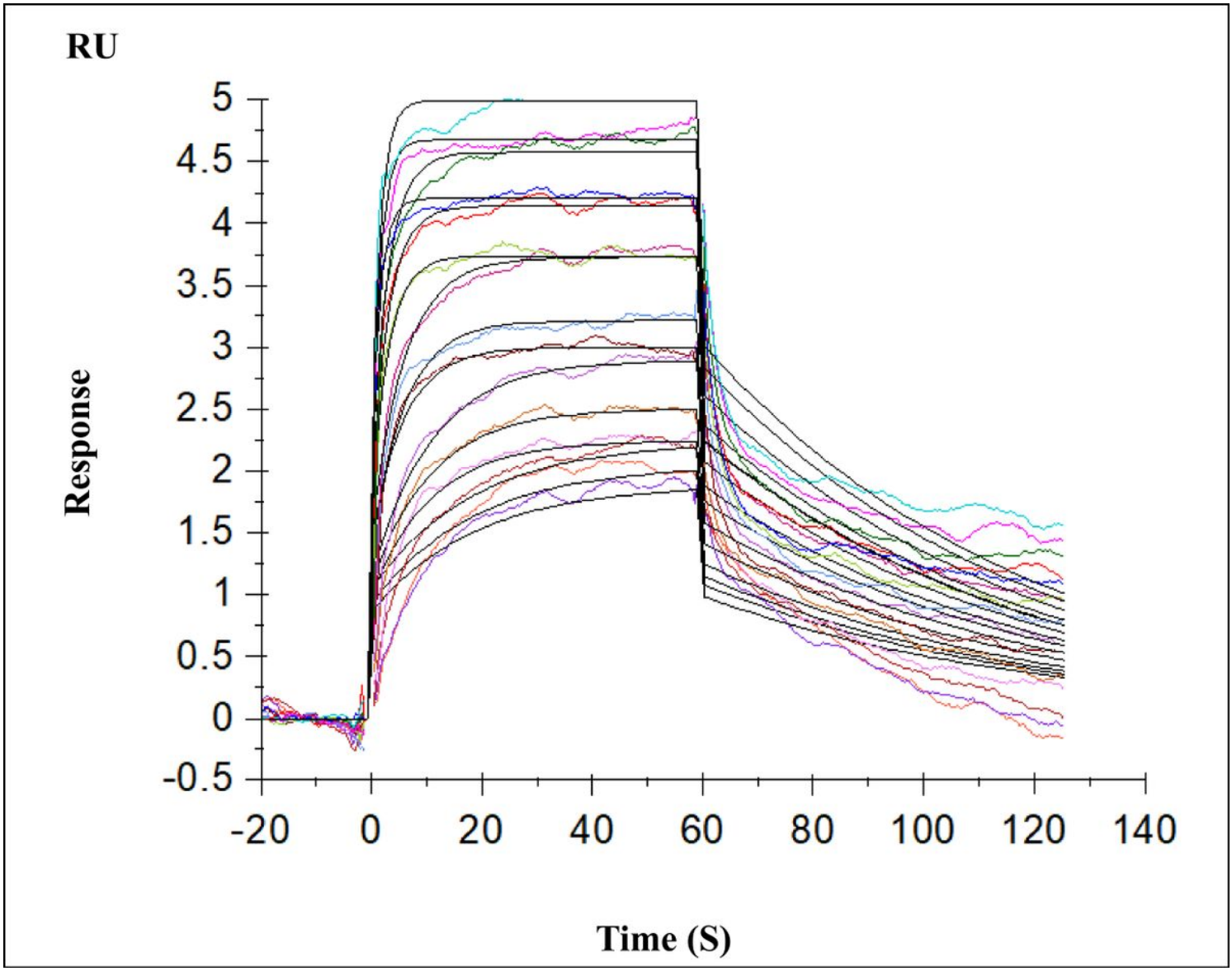

Figure 10

Caption kinetic analysis for the binding of different analyte concentrations (1000nM, 500nM, 250nM, 125nM, 62.5nM and 0nM) with anti mAb-EGFR/CR2RK as ligand using 1:1 binding model