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이학박사 학위논문

Development of competition ELISA for N-terminal fragment of proBNP (NT-proBNP) using peptide mimotope and bispecific antibody against NT-proBNP and cotinine

펩타이드 미모톱과 이중특이성 항체를 이용한 NT-proBNP 의 경쟁적 효소면역측정법 개발

2014 년 8 월

서울대학교 대학원

협동과정 종양생물학 전공

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지도 교수 정 준 호

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Development of competition ELISA for N-terminal fragment of proBNP (NT-proBNP) using peptide mimotope and bispecific antibody against NT-proBNP and cotinine

by

Yujean Lee

A Thesis Submitted to the Interdisciplinary Graduate Program in partial Fulfillment of the Requirement of the Degree of Doctor of Philosophy in Cancer Biology at Seoul National University

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Approved by Thesis Committee:

Professor _____ **Chairman**

Professor _____ **Vice chairman**

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Abstract

N-terminal fragment of proBNP (NT-proBNP) is an important biomarker for diagnosis of congestive heart failure. Although biological function of NT-proBNP is not well known, it has many benefits as a biomarker. NT-proBNP has several *O*-linked glycosylation sites indicating that circulating NT-proBNP in human blood is also glycosylated. It is quite difficult to develop an antibody that exhibits glycosylation-independent binding. In addition, N- and C-terminally truncated NT-proBNP, cleaved by physiological proteolysis, was identified. The recombinant NT-proBNP-Fc fusion protein was glycosylated like as circulating NT-proBNP in human blood. NPBR9, monoclonal anti-NT-proBNP antibody, was generated from rabbit immune libraries and it was modified to bispecific scFv-C_{κ} fusion protein and IgG_1. Characterization of the binding sites was identified by phage display of a random peptide library and site-directed mutagenesis. Its epitope was located close to C-terminal region of NT-proBNP rather than extreme C-terminus. Furthermore, the epitope of NPBR9 has the consensus sequence containing three amino acids, "$H_{64}R_{65}K_{66}$" in NT-proBNP. We also identified that substitution of G_{63} or K_{66} in NT-proBNP completely abolished its reactivity to the antibody. In addition, peptide mimotopes were chemically synthesized with –GGGSC linker at the C-terminal end, and they were competed with the recombinant NT-proBNP-Fc fusion protein in the competition immunoassay. In this study, we aimed to develop an antibody reactive to an epitope that is relatively less influenced by

glycosylation and N- and C-terminal cleavage. Then we performed a competition enzyme immunoassay using this antibody and epitope mimetic peptides. And, through sandwich enzyme immunoassay, the reactivity of this antibody to both recombinant and de-glycosylated NT-proBNP were confirmed to be quite similar. However, this competition enzyme immunoassay was not enough to sensitive detection for lower concentration of NT-proBNP. Hence, we applied NPBR9 as detection antibody in sandwich immunoassay, because NPBR9 is less influenced by glycosylation and N- and C-terminal cleavage. Therefore, it may be overcome of inadequate to detection of glycosylated or truncated NT-proBNP. In conclusion, we report that an antibody reactive to Gly_{63}–Lys_{68} of NT-proBNP exhibits *O*-glycosylation-independent binding, and its reactivity to NT-proBNP whether glycosylated or not was confirmed by sandwich enzyme immunoassay. Finally, we developed a competition enzyme immunoassay using this antibody and peptide mimotopes.

Keywords: NT-proBNP, N-terminal fragment of proBNP; *O*-linked glycosylation; phage display; peptide mimotope; enzyme immunoassay

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Introduction

1. N-terminal fragment of proBNP (NT-proBNP)

The natriuretic peptide family includes atrial natriuretic peptide (ANP), brain (or B-type) natriuretic peptide (BNP), and C-type natriuretic peptide (CNP). All NPs are important regulators as diuresis, natriuresis, and vasolidation (1).

BNP was first isolated from porcine brain (2). However it was later identified that BNP was predominantly synthesized from the heart ventricles (3, 4), so it also known as B-type natriuretic peptide. In case of ANP is secreted from the heart atria (5), and CNP is mainly produced in the central nervous system and vascular endothelium (6).

All natriuretic peptides are synthesized as prohormones. In case of BNP, it is synthesized within the cardiomyocytes as a pre-proBNP (134 amino acids). Pre-proBNP is released from cardiomyocytes into the blood when the ventricular wall is stretched. In the blood, proBNP is cleaved to yield mature functional BNP (32 amino acids) and the N-terminal fragment of proBNP (NT-proBNP, 76 amino acids) by proprotein convertase such as corin and furin **(Fig. 1A)** (7, 8).

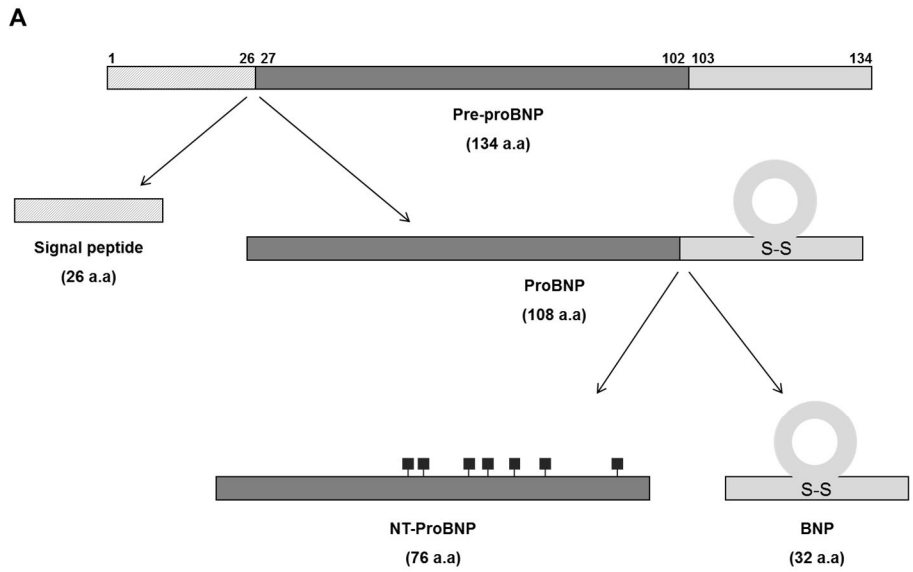
Natriuretic peptides (NPs) are recognized as useful biomarkers in the diagnosis of congestive heart failure (CHF). Although NP levels depend on

the age and gender of the patient, these levels can drastically change based on the severity of CHF severity (8, 9). As the plasma concentration of BNP is most closely related with heart failure, BNP has been used as the biomarker more frequently among NPs (10). The biological function of NT-proBNP is unknown; however, it has advantages of a biomarker. The circulating level in blood and *in vitro* stability of NT-proBNP are higher than those of BNP **(Table 1)** (11). Therefore NT-proBNP became more frequently measured than BNP in the diagnosis of CHF.

1.1. Glycosylation of proBNP

Recombinant proBNP which expressed in Chinese hamster ovary (CHO) cells and native proBNP circulating in human blood retains an *O*-linked glycosylation (12). Approximately 10% of the amino acid residues of NT-proBNP are reported to be glycosylated (12-15). The residues potentially glycosylated in NT-proBNP are Thr_{36}, Ser_{37}, Ser_{44}, Thr_{48}, Ser_{53}, Thr_{58}, and Thr_{71} **(Fig. 1B)**. All these residues are complete glycosylation sites except Thr_{36} and Thr_{58} (12). The physiological reason for glycosylation of proBNP is not fully understood; however, it is likely to increase the stability of the peptide. In addition, these glycosylation patters affect the recognition of NT-proBNP by antibodies (15). When antibodies were expressed in *E. coli* that originally non-glycosylated, these antibodies could not recognized to epitopes consisting of residues 28–45, 31–39, 34–39, and 46–56 in glycosylated NT-

proBNP extracted from pooled plasma of heart failure patients compared with the de-glycosylated form (13). In the development of antibodies for diagnostic assay, antibodies binding to NT-proBNP irrespective of its glycosylation patterns are preferred.



B

1 11 21 31 41
HPLGSPGSAS DLETSGLQEQ RNHLQGKLSE LQVEQ**T**SLEP LQE**S**PRP**T**GV

51 61 71
WK**S**REVA**T**EG IRGHRKMVLY **T**LRAPR

Figure 1. Scheme of proBNP processing (16) and the sequence of NT-proBNP. (A) Pro-BNP is synthesized within cardiomyocytes as a pre-proBNP and it is cleaved to NT-proBNP and BNP when myocyte stretched. *O*-glycosylation sites in NT-proBNP are marked as dark boxes (12). (B) The peptide sequence of NT-proBNP. *O*-linked glycosylation sites are marked by underlined and bold (Underlined, complete glycosylation sites; Bold, partial glycosylation sites). The arrows show physiologically proteolysis sites.

Table 1. Advantages of NT-proBNP as a CHF biomarker (17, 18).

	NT-proBNP	BNP
Biological characteristics	Inactive	Active
Half life	60 – 120 min	20 min
In vitro stability	3 days (at least)	Within 24 hr
Plasma level	Concentration of NT-proBNP is 10-folds higher than that BNP's	

NT-proBNP, N-terminal fragment of proBNP; BNP, Brain (B-type) natriuretic peptide

1.2. Proteolysis of proBNP

Circulating BNP in human blood are deleted 2 amino acid residues from the amino terminus when it was incubated at room temperature during overnight, and the cleaved peptides were analyzed by gel-filtration HPLC (19). In this result, the peptide was identified truncated BNP, BNP_{3-32}. The peptide was cleaved by dipeptidyl-peptidase IV (DPP IV/CD26). It was preferentially cleaves dipeptidase with a proline or alanine in the second position in the second amino terminal residues (20). Interestingly, other report suggested that proBNP also consist a proline in the second position, it may be susceptible to DPP-IV (21) .

Recently it was reported that N- and C- termini of NT-proBNP are cleaved in the blood by physiological proteolysis (22). To identify the cleavage sites and major proteolytic products of NT-proBNP in the circulation, NT-proBNP molecules were collected through immunoprecipitation (IP) from heart failure patients. Afterward enriched plasma NT-proBNP was digested with trypsin and analyzed by liquid chromatography (LC)-electrospray ionization-tandem mass spectrometry (MS/MS). In this result, several peptides were identified which were tryptic or semitryptic peptides. However some peptide fragments which do not have trypsin recognition sites also identified in this results. It means that proteolysis frequently occurred between Pro_2–Leu_3, Leu_3–Gly_4, Pro_6–Gly_7 and Pro_{75}–Arg_{76} on NT-proBNP. In this report shows that antibodies which bind to extreme N- or C- termini measured a low concentration of

circulating NT-proBNP through AlphaLISA immunoassay. The antibodies targeting nonglycosylated and nonterminal epitopes measured a higher concentration of NT-proBNP. In this results demonstrated that circulating NT-proBNP was mixed with several truncated NT-proBNP. Therefore detection of N- and C- cleaved fragment of NT-proBNP is another hurdle in the development of immunoassay.

1.3. Commercial immunoassay for NT-proBNP

There are several clinically available sandwich immunoassays. They used one pair of antibodies for detection of NT-proBNP **(Fig. 2 and Table 2)**. One assay employs polyclonal antibodies against epitopes of NT-proBNP$_{1-21}$ and NT-proBNP$_{39-50}$ (Roche) (23). Another polyclonal antibody pair that recognizes NT-proBNP$_{8-29}$ and NT-proBNP$_{31-57}$ is used in another assay kit (Biomedica) (24). Monoclonal antibody pairs recognizing NT-proBNP$_{13-27}$ and NT-proBNP$_{61-76}$ or NT-proBNP$_{1-21}$ and NT-proBNP$_{61-76}$ are also employed (HyTest) (14, 25). These sandwich immunoassays are weak for sensitive detection to glycosylated or fragmented from both ends of NT-proBNP, because these antibodies are recognized on glycosylated or extreme N- or C-termini.

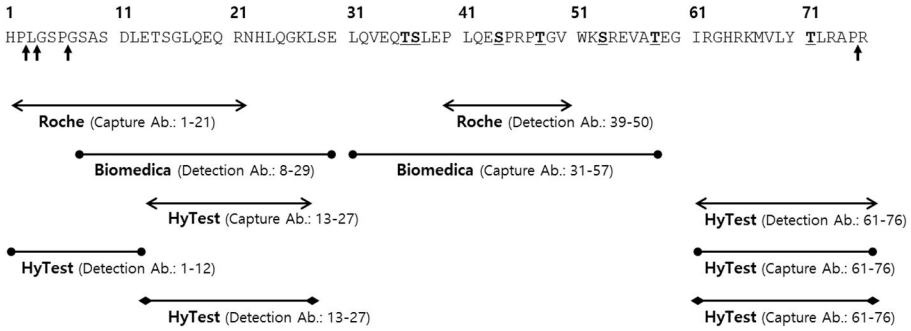


Figure 2. Antibody recognition sites of commercial immunoassays.

Several commercial immunoassays used one pair of antibodies for detection of NT-proBNP. It is hard to designate of antibody recognition sites on NT-proBNP which rule out of glycosylation sites or both termini.

Table 2. Commercial immunoassays for NT-proBNP (13, 23, 24)

Company	Assay type	Detection	Antibodies	Limit of detection (LOD)
Roche	Sandwich immunoassay	Electrochemiluminoscence	Polyclonal Ab.	1 pg/mL
Biomedica	Sandwich immunoassay	Colorimetric	Polyclonal Ab.	25 pg/mL
HyTest	Sandwich immunoassay	Electrochemifluorescence	Monoclonal Ab.	10~15 pg/mL

2. Phage display

Phage display technology is powerful tool for the selection of specific monoclonal antibodies against known or novel antigen. The filamentous bacteriophages (phage) are viruses contain a circular single-stranded DNA and Ff class of the phages like as M13 and Fd are well known. The name shows that these phage are infected to their host, *E. coli*, through the F pilus (26). The phages mainly enclosed gene 8 protein that was major coat protein, and other four coat proteins are also located on phage surface. Among these coat proteins, the inserted genes were displayed through gene 3 protein (pIII), gene 6 protein (pVI) or gene 8 protein (pVIII). However phage DNA has low transformation efficiency, so phagemid vector used to phage display system for simple cloning directly into phage genome and displayed large proteins on phage. Phagemid display on pIII is the most commonly used and foreign protein is presented on the phage surface through insertion of foreign DNA before gene 3 by restriction sites **(Fig. 3A)** (27).

The ultimate purpose of phage display is the selection of specific antibodies to target antigen and excluded nonspecific phages through bio-panning. Bio-panning consists of 4 steps; 1) immobilized target antigen and incubated with phage libraries, 2) wash out unbound or low affinity phages, 3) elute bound phages, 4) overnight amplification of eluted phages. All steps are repeated three or four times until enrichment is achieved **(Fig. 3B)**. After final round of biopanning, colony picking of individual clones and infected to *E.*

coli cells for phage amplification of each clones. Amplified each phage clones are screened by phage enzyme immunosorbent assay (ELISA). Antibody selection procedures are on-going through sequencing analysis, over-expression in mammalian cells and antibody purification by affinity chromatography.

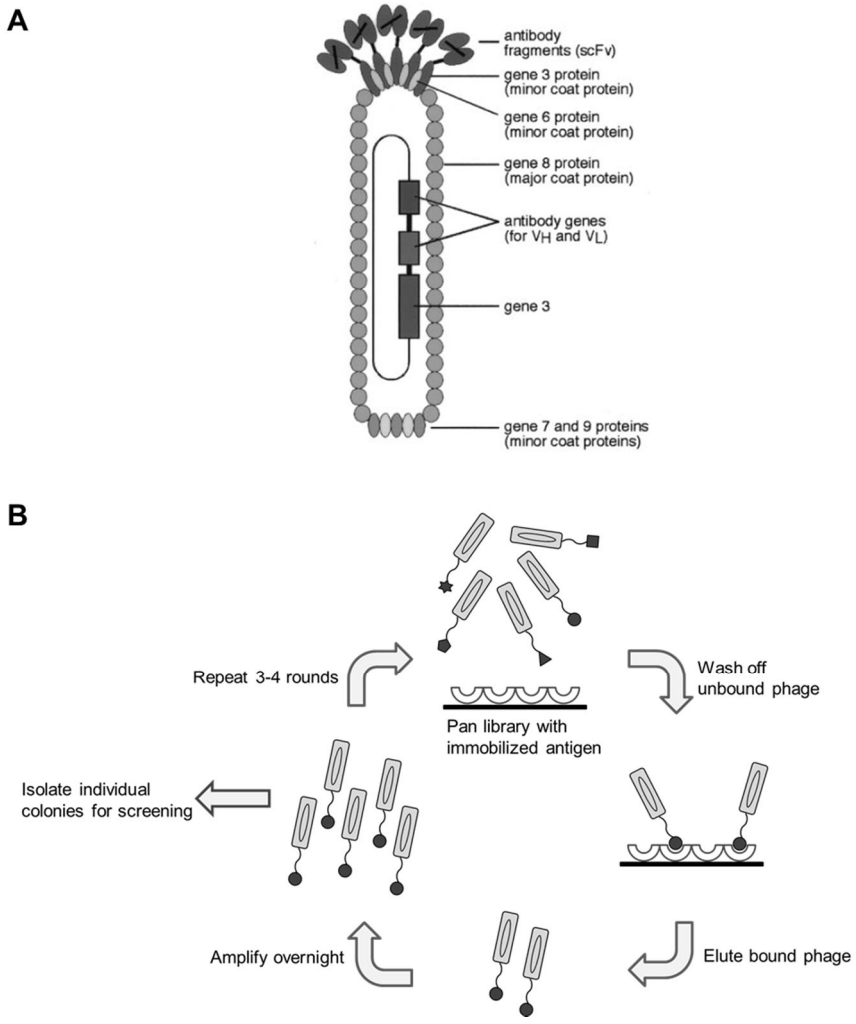


Figure 3. Overview of phage display systems. (A) Structure of filamentous phage displaying scFv fragment on pIII (27). (B) Experimental scheme for bio-panning.

3. Purpose of this study

In this context, it is quite difficult to develop an antibody pair that is reactive to epitopes not influenced by glycosylation or N- and C- terminal cleavage. In this study, we aimed to develop an antibody reactive to an epitope relatively less influenced by glycosylation and N- and C-terminus cleavage. First, we established a mammalian expression system for NT-proBNP, and confirmed that the purified recombinant protein is glycosylated by digestion with *O*-glycosidase and neuraminidase in gel electrophoresis. Next, using phage display of a combinatorial rabbit antibody library, we generated an antibody with reactivity to NT-proBNP independent of glycosylation. Furthermore, we defined the epitope using the phage display of a random peptide library, which turned out to be Gly_{63}–Lys_{68}. This region is expected uninfluenced by glycosylation and protected from C-terminal cleavage.

Finally a competition enzyme immunoassay was developed using this antibody and epitope-mimetic peptide. Furthermore, we confirmed the reactivity of this antibody which *O*-glycosylation-independent binding to both recombinant and de-glycosylate NT-proBNP through sandwich enzyme immunoassay.

Materials and Methods

1. Preparation of NT-proBNP-Fc fusion protein

1.1. Total RNA isolation, RT-PCR and sub-cloning

Total RNA was prepared from HEK (Human Embryonic Kidney) cells using TRIzol® reagent (Invitrogen, Carlsbad, CA). Subsequently, cDNA was synthesized using the Superscript™ III first-strand synthesis supermix with Oligo (dT) priming (Invitrogen). The human NT-proBNP gene was amplified using the forward and reverse primers, and the primer sequences for the human NT-proBNP were as follows: 5'-GGC CGG CCC AGG CGG CCC ACC CGC TGG GCA GCC-3', and 5'-GGC CGG CCC CAC CGG CCC CTC GTG GTG CCC GCA GGG T-3'. The reactions were carried out under the following conditions: preliminary denaturation at 95°C for 5 min, followed by 35 cycles of 30 sec at 94°C, 30 sec at 55°C, and 90 sec at 72°C. The reaction was ended in 10 min at 72°C for a final extension. The gene was subcloned into the modified pCEP4 vector, which has two *Sfi*I sites for insertion of DNA fragment and human Fc tag after *Sfi*I site, as described previously (28).

1.2. Cell culture, transient expression and purification

For overexpression of the recombinant NT-proBNP-Fc fusion protein, the expression vector was transfected into HEK293F cells (1.0×10^6 cells/mL) (Invitrogen) using polyethylenimine (PEI: Polysciences, Warrington, PA), and the transfected cells were cultured in FreeStyle™ 293 expression medium (Invitrogen) in a 37°C CO_2 incubator as described previously (29). The recombinant NT-proBNP-Fc fusion protein was purified by affinity chromatography using protein A sepharose (Repligen Corp., Cambridge, MA) column, as described previously (28).

2. Generation of monoclonal anti-NT-proBNP antibodies

2.1 Immunization

Two New Zealand white rabbits were immunized with the purified recombinant NT-proBNP-Fc fusion protein. Briefly, 10 μg of the purified NT-proBNP-Fc fusion protein in 2 mL of Freund's adjuvant (Sigma, St. Louis, MO) was incubated at 37°C for 30 min, and used for immunization of New Zealand white rabbits. The immunization was performed 4 times at 3 week intervals, as described previously (30). The titer of serum antibody was determined by ELISA using horseradish peroxidase (HRP) conjugated mouse anti-rabbit IgG polyclonal antibody (Pierce, Rockford, IL) as secondary probe.

2.2. Isolation of total RNA from the spleen and bone marrow of the immunized rabbits

Total RNA was isolated from the spleen and bone marrow of the immunized rabbits using TRIzol® reagent (Invitrogen). The extracted spleen and bone marrow in TRIzol® reagent was homogenized using homogenizer. The homogenized samples were centrifuged at 2,500 g for 10 min at 4°C. The supernatant was transferred to a 50 mL centrifuge tube and added 1 mL of BCP (1-bromo-3-chrolo-propane) (Sigma) to each supernatant. The tubes were vortexed for 15 sec and incubated for 15 min at room temperature. The mixture was centrifuged at 17,500 g for 15 min at 4°C and the supernatant was transferred to fresh tube. The 15 mL of isopropanol was added and incubated for 10 min at room temperature. Repeated centrifugation as described above, the supernatant was removed carefully and the pellet was washed with 10 mL of 75% ethanol without resuspension. After centrifugation at 17,500 g for 10 min at 4°C, the supernatant was removed and the pellet was air-dried briefly at room temperature. The pellet was dissolved in 250 μL of RNase-free water and stored in -80°C. The RNA concentration was determined by measuring the optical density (OD) at 260 nm (40 ng/μL RNA gives and OD_{260}=1) and the purity was calculated by the ratio of OD_{260}/OD_{280} (typically in the range of 1.6 to 1.9).

2.3. First-strand cDNA synthesis from total RNA

First-strand cDNA was synthesized using the Superscript™ III first-strand synthesis supermix with Oligo (dT) priming (Invitrogen) following the manufacturer's insturctions. Briefly, 5 μg of the isolated total RNA was mixed with 1 μL of 50 μM oligo$(dT)_{20}$, 1 μL of 10 mM dNTP mix and added DEPC (diethyl pyrocarbonate)-treated water up to 10 μL. The mixture was incubated for 5 min at 65 °C and placed on ice for at least 1 min. Next, 2 μL of 10X reaction buffer, 4 μL of 25 mM $MgCl_2$, 2 μL of 0.1M DTT (dithiothreitol), 1 μL of RNaseOUT^{TM} (40 U/μL), and 1 μL of Superscript III reverse transcriptase were added to the mixture and incubated for 50 min at 50°C. The reaction was terminated by incubation for 5 min at 85°C and chilled on ice. Finally, 1 μL of RNaseH was added and incubated for 20 min at 37°C. The first-strand cDNA was stored at -20°C utile use.

2.4. First round of PCR

The cDNAs from bone marrow and spleen of the immunized rabbits were amplified by PCR using expand high fidelity PCR system (Roche molecular diagnostics, Pleasanton, CA). Ten primer combinations for amplification of rabbit V_L ($9\times V_\kappa$ and $1\times V_\lambda$), and 4 combinations for amplification of rabbit V_H coding sequences were used together **(Fig. 4 and Table 3)**. About 0.5 μg of cDNA was mixed with 60 pmol of each primer sets, 10 μL of 10X reaction buffer, 8 μL of 2.5 mM dNTPs (Promega, Madison, WI), 0.5 μL of Taq DNA

polymerase, and distilled water to bring the final volume of 100 μL. The reactions were carried out under the following conditions: 30 cycles of 15 sec at 94°C, 30 sec at 56°C, and 90 sec at 72°C, followed by a final extension for 10 min at 72°C. The size of V_L and V_H products was about 350 base pair, each PCR products were analyzed on a 2% agarose gel. The light chain PCR products were combined into one pool and the heavy chain products into another pool. They were loaded and run on a 1.5% agarose gel and purified by QIAEX II gel extraction kit (Qiagen, Valencia, CA). The purified PCR products were quantified by reading the OD at 260 nm.

2.5. Second round of PCR

For the generation of scFvs, the first round V_L products and V_H products were combined by overlap extension PCR **(Fig. 5)**. The primer sets for this were described as above **(Table 3)**. Each 100 ng of purified light chain product and heavy chain product were mixed with 60 pmol of each primer, 10 μL of 10X reaction buffer, 8 μL of 2.5 mM dNTPs (Promega), 0.5 μL of Taq DNA polymerase, and water to bring the final volume of 100 μL. The reactions were carried out under the following conditions: 20 cycles of 15 sec at 94°C, 30 sec at 56°C, and 2 min at 72°C, followed by a final extension for 10 min at 72°C. About 700 base pair sized products were loaded and run on a 1.5% agarose gel. They were purified as described above.

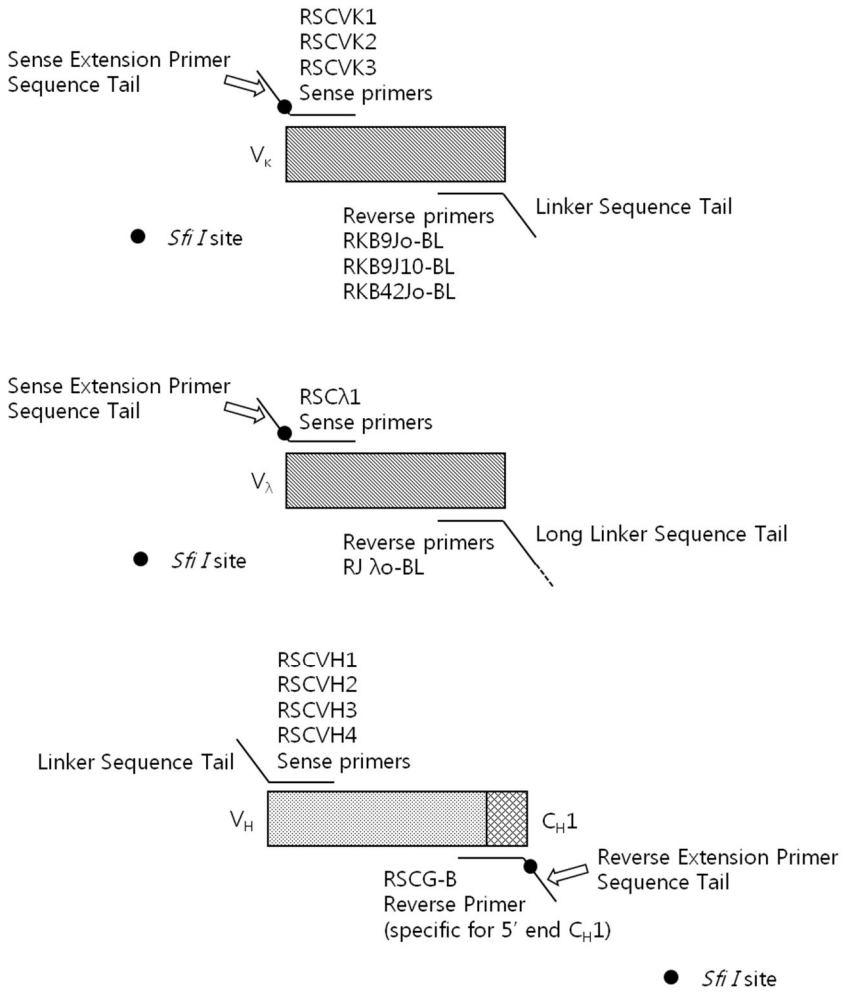


Figure 4. The amplification of rabbit V_κ, V_λ, and V_H sequences for the construction of scFv libraries. Each sense primer is combined with each reverse primer to amplify rabbit V_κ, V_λ, and V_H gene segments from cDNA. The sense primers of V_κ, V_λ, and the reverse primers of V_H have *Sfi*I sites on sequence tail. Theses *Sfi*I sites are used to subcloning into phagemid vector. The primers which have linker sequence tail are used in the overlap extension PCR.

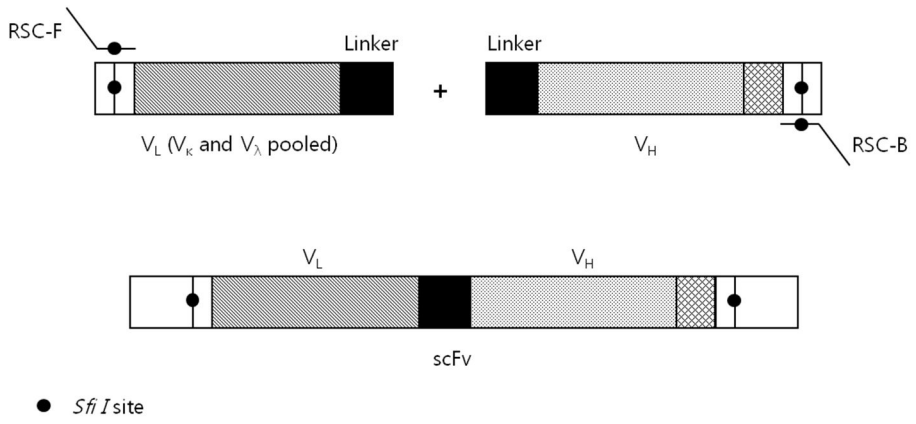


Figure 5. Overlap extension PCR to combine the rabbit V_L and V_H fragments for the construction of scFv libraries (long linker). The sense primer (RSC-F) and reverse primer (RSC-B) used in overlap extension PCR. They recognized the sequence tails that were generated from the first round PCR.

Table 3. Primers for the construction of rabbit scFv libraries

V_κ 5' Sense Primers (5' → 3')	
RSCVK1	GGG CCC AGG CGG CCG AGC TCG TGM TGA CCC AGA CTC CA
RSCVK2	GGG CCC AGG CGG CCG AGC TCG ATM TGA CCC AGA CTC CA
RSCVK3	GGG CCC AGG CGG CCG AGC TCG TGA TGA CCC AGA CTG AA
V_κ 3' Reverse Primers (5' → 3')	
RKB9JIo-BL	GGA AGA TCT AGA GGA ACC ACC CCC ACC ACC GCC CGA GCC ACC GCC ACC A GA GGA TTT GAT TTC CAC ATT GGT GCC
RKB9Jo-BL	GGA AGA TCT AGA GGA ACC ACC CCC ACC ACC GCC CGA GCC ACC GCC ACC A GA GGA TAG GAT CTC CAG CTC GGT CCC
RKB42Jo-BL	GGA AGA TCT AGA GGA ACC ACC CCC ACC ACC GCC CGA GCC ACC GCC ACC A GA GGA TTT GAC SAC CAC CTC GGT CCC
V_λ 5' Sense Primer (5' → 3')	
RSCλ1	GGG CCC AGG CGG CCG AGC TCG TGC TGA CTC AGT CGC CCT C
V_λ 3' Reverse Primer (5' → 3')	
RJλo-BL	GGA AGA TCT AGA GGA ACC ACC CCC ACC ACC GCC CGA GCC ACC GCC ACC A GA GGA GCC TGT GAC GGT CAG CTG GGT CCC
V_H 5' Sense Primers (5' → 3')	
RSCVH1	GGT GGT TCC TCT AGA TCT TCC CAG TCG GTG GAG GAG TCC RGG
RSCVH2	GGT GGT TCC TCT AGA TCT TCC CAG TCG GTG AAG GAG TCC GAG
RSCVH3	GGT GGT TCC TCT AGA TCT TCC CAG TCG YTG GAG GAG TCC GGG
RSCVH4	GGT GGT TCC TCT AGA TCT TCC CAG SAG CAG CTG RTG GAG TCC GG
V_H 3' Reverse Primer (5' → 3')	
RSCG-B	CCT GGC CGG CCT GGC CAC TAG TGA CTG AYG GAG CCT TAG GTT GCC C
Overlap Extension Primers (5' → 3')	
RSC-F (sense)	GAG GAG GAG GAG GAG GAG GCG GGG CCC AGG CGG CCG AGC TC
RSC-B (reverse)	GAG GAG GAG GAG GAG GAG CCT GGC CGG CCT GGC CAC TAG TG

2.6. Restriction enzyme digestion of the purified overlap extension product and the vector DNA

The PCR product and the pComb3X vector for cloning were digested with *Sfi*I (Roche). Briefly, 10 μg of the purified overlap PCR product was mixed with 360 U of *Sfi*I (16 U per μg of DNA) (Roche), 20 μL of 10X reaction buffer M, and water to bring the final volume of 200 μL. In parallel, 20 μg of pCobm3X vector was incubated with 120 U of *Sfi*I (6 U per ug of DNA) (Roche), 20 μL of 10X reaction buffer M, and water to bring the final volume of 200 μL. Both digest were incubated for 5 h at 50°C. About 700 base pair of the digested insert was purified on a 1% agarose gel and the vector (~3400 base pair) and the stuffer fragment (~1600 base pair) were purified on a 0.6% agarose gel as described above.

2.7. Preparation of electrocompetent *E. coli*

A single *E. coli* colony on a LB plate was inoculated to 15 mL of prewarmed SB medium in a 50 mL polypropylene tube and grown overnight at 250 rpm and 37 °C. The culture was diluted 200 folds into a 2 L flask with 500 mL of SB and mixed with 10 mL of 20% (w/v) glucose, and 5 mL of 1M $MgCl_2$. The culture was shook at 250 rpm and 37°C until the OD at 600 nm was about 0.8-0.9. After the proper OD was reached, the culture was divided into two pre-chilled 500 mL centrifuge bottles and centrifuged at 3,000 g for 20 min at 4°C. The supernatant was discarded and the bacterial pellet was

resuspended in 250 mL of pre-chilled 10% (v/v) glycerol. The resuspended pellet was centrifuged as before, and washed three times with 10% (v/v) glycerol. The cells were resuspended in 300 μL and immediately aliquot into the microcentrifuge tubes, and stored at -80°C until use.

2.8. Preparation of VCSM13 helper phage

ER2738 (New England Biolabs, Beverly, MA) cells were inoculated in 10 mL of SB medium and shook at 250 rpm and 37°C until the OD at 600 nm was between 0.8 and 0.9. A single VCSM13 helper phage plaque on a LB plate was added to the culture for infection. The phage infected cells was grown for 2 h at 37°C, and transferred to 500 mL centrifuge bottle containing 200 mL of prewarmed SB medium. Kanamycin (50 mg/mL) was added to the culture and shook overnight at 250 rpm and 37°C. The culture was centrifugated at 3,000 g for 15 min and the supernatants were incubated in a water bath at 70°C for 20 min. After centrifuged at 3,000 g again for 15 min, the supernatants were transferred to fresh 50 mL polypropylene tubes and stored at 4°C.

2.9. Ligation of the digested overlap PCR product with the vector DNA

2.9.1. Test ligations

Small scale ligations were performed to assess the suitability of the vector and inserts for high efficiency ligation and transformation. *Sfi*I digested vector DNA (140 ng) was mixed with 70 ng of *Sfi*I digested PCR products, 4 μL of 5X ligase buffer, 1 μL of T4 DNA ligase (Invitrogen), and water to bring the final volume of 20 μL. The ligation efficiency was compared to two controls, for insert control and for vector self-ligation. For insert control, 140 ng of *Sfi*I digested stuffer fragment used for instead of insert. Only *Sfi*I digested vector DNA was mixed with 5X ligase buffer and 1 μL of T4 DNA ligase for vector self-ligation control. The ligation mixtures were incubated between 4 h and overnight at 16°C. One microliter of ligation mixture was mixed with 50 μL of ER2738 (NEB) electrocompetent cells and incubated on ice for 1 min. The mixture was transferred into 0.2 cm cuvette and eletroporated by Gene pulser (Bio Rad laboratories, Hercules, CA) at a condition of 2.5 kV, 25 μF and 200 Ω. The cells were resuspended with 3 mL of SB medium and incubated for 1 h at 37°C. The transformed cultures were diluted 10 fold and 100 fold with prewarmed SB medium, and plated 100 μL of each dilution on LB plates with carbenicillin for calculation of transformants. The final library size should be at least 10^8 CUF per μg of vector DNA and should have less than 5% vector self-ligation.

2.9.2. Library ligation and transformation

Single library ligation was carried out using 1.4 μg of *Sfi*I digested pComb3X vector, 700 ng of *Sfi*I digested PCR product, 40 μL of 5X ligase buffer, 10 μL of T4 DNA ligase, and water to bring the final volume of 200 μL. The ligation mixture was incubated overnight at 16°C. The mixture was precipitated by adding 1 μL of glycogen, 20 μL (0.1 volumes) of 3 M sodium acetate, pH 5.2, and 440 μL (2.2 volumes) of ethanol. The sample was stored at -80°C followed by centrifugation at full speed for 15 min at 4°C. The pellet was rinsed with 1 mL of 70% (v/v) ethanol and dried briefly, and dissolved in 15 μL of distilled water. The ligated library sample was transformed into 300 μL of ER2738 (NEB) electrocompetent cells as described above. The cells was resuspended with 5 mL of SB medium and incubated for 1 h at 37°C. 10 mL of prewarmed SB medium and 3 μL of 100 mg/mL carbenicillin were added to the culture. To titer the transformed library, the culture was diluted 100 fold in SB medium, and plated 10 μL and 100 μL of this on LB plates with carbenicillin. The culture was incubated 1 h at 37°C and 4.5 μL of 100 mg/mL carbenicillin was added to the culture and shook for an additional hour. The culture was added with 2 mL of VCSM13 helper phage (10^{12} to 10^{13} pfu/mL), 183 mL of prewarmed SB medium, and 92.5 μL of 100 mg/ml carbenicillin. This culture was shook at 300 rpm for 1.5–2 h at 37°C. Kanamycin (50 mg/mL, 280 μL) was added and continued shaking overnight at 37°C. In the next day, the phage pool was collected by centrifugation at 3,000 g for 15 min. The supernatant was transferred to a clean 500 mL

centrifuge bottle and the bacterial pellet was saved for phagemid DNA preparation. 8 g of polyethylene glycol-8000 (PEG-8000) and 6 g of NaCl were mixed with supernatant. The solid was dissolved well by shaking at 37°C for 10 min and the mixture was stored on ice for 30 min. The phage was pelleted by centrifugation at 15,000 g for 15 min and 4°C. The supernatant was discarded and the phage pellet was resuspended in Tris-buffered saline (TBS) containing 1% (w/v) bovine serum albumin (BSA) supplemented with 0.02% NaN_3. The phage sup was centrifugated and filtered with 0.2 μm filter for eliminate the remnants of bacterial cells.

2.10. Biopanning

To select specific binders from the library, a total of five rounds of biopanning were performed as described previously (31) . Briefly, 3 μg of NT-proBNP prepared from *E. coli* (Scipac, Kent, UK) was conjugated with 5.0×10^6 paramagnetic beads (DynaBeads, Invitrogn) following the guidelines provided by the supplier. After overnight incubation at 37°C on a rotator, the beads were washed four times with 500 μL of 0.5% BSA in PBS (w/v) and blocked with 100 μL of 3% BSA in PBS (w/v). The phage library (100 μL) was added to beads, followed by incubation for 2 h at 37°C. The beads were washed once with 500 μL of 0.05% Tween 20 in PBS (v/v, PBST), and the number of washes was increased in subsequence rounds: three times for the second and third rounds and five times for the last round, respectively (28).

Through this step, unbound or weakly bound phages were removed. Thereafter, 50 μL of 0.1 M glycine-HCl (pH 2.2) was added to the beads and the beads were incubated for 10 min at 37°C on a rotator. The eluate was neutralized with 4 μL of 2 M Tris-Cl (pH 9.1), and repeat the elution step. The scFv-displaying phages were infected to 2 mL of *E. coli* ER2738 (NEB) cells, and then rescued by adding helper phages during overnight as described above. Individual phage clones were selected from the output titration plate from the last round of biopanning. In the next day, phage pool was collected by PEG-8000 and NaCl as described above.

2.11. Selection and identification of clones

2.11.1. Small scale phage amplification

Individual clones on LB plates were inoculated in deep well plate (Axygen, Union city, CA) containing 1 mL of SB medium with 50 μg/mL carbenicillin. The culture was shook at 250 rpm and 37°C until the OD at 600 nm was about 0.6. Next, 10 μL of VCSM13 helper phage (10^{12} to 10^{13} pfu/mL) was added, and followed by incubation for 2 h at 37°C. Seven microliters of 50 mg/mL kanamycin was added and continued shaking overnight at 37°C.

2.11.2. Phage ELISA

The microtiter plates (Corning Costar Corp., Cambridge, MA) were coated with the recombinant NT-proBNP-Fc fusion protein (10 μg/mL in PBS, 25 μL) overnight at 4°C. The wells were blocked with 150 μL of 3% BSA in PBS (w/v) for 1 h at 37°C. The plates were incubated with phage-containing culture supernatants that equally mixed with 6% BSA in PBS (w/v) at 37°C for 2 h and washed three times with 150 μL of 0.05% PBST. Then HRP-conjugated anti-M13 antibody (GE Healthcare, Piscataway, NJ) diluted in blocking buffer (1:5,000) was added to each well, and the plate was incubated for 1 h at 37 °C. After washing three times with 150 μL of 0.05% PBST, 50 μL of 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid (ABTS) substrate solution (Pierce) for 30 min at 37°C. Subsequently, the absorbance of each well was measured at 405 nm using a plate reader (Labsystems S.L., Barcelona, Spain).

2.11.3. Sequencing analysis

To identify the selected scFvs from phage ELISA, the nucleotide sequences of clones were determined by sequencing analysis. The primer sequences for pComb3X clones are as follow: 5'-ACA CTT TAT GCT TCC GGC TC-3'.

3. NT-proBNP de-glycosylation

The purified recombinant NT-proBNP-Fc fusion protein was treated with *O*-glycosidase (40,000,000 U/mL) (NEB) and neuraminidase (50,000 U/mL) (NEB) in 50 mM sodium phosphate buffer (pH 7.5) containing 1% NP-40 at 37°C for 3 h. After incubation, the de-glycosylated NT-proBNP-Fc fusion protein was subjected to SDS-polyacrylamide gel electrophoresis and Coomassie blue staining.

4. SDS PAGE and immunoblotting

All experiments were performed in the denaturing reaction conditions. The recombinant NT-proBNP-Fc fusion protein (100 ng) was dissolved in 12 μL of LDS sample buffer (Invitrogen) with 10% reducing agent (Invitrogen). The denatured recombinant NT-proBNP-Fc fusion protein was subjected to two sets of 4-12% Bis-Tris SDS-polyacrylamide gel electrophoresis (Invitrogen). One gel was stained with Coomassie brilliant blue, and the other gel was transferred to a Whatman™ Protran® nitrocellulose membrane (GE Healthcare) for immunoblotting. The membrane was divided into three parts, and blocked with 5% skim milk at 25°C for 1 h. Two membranes were sequentially incubated with 1 μg/mL of each IgG_1 in blocking buffer, and HRP-conjugated anti-human Fab antibody (Pierce) diluted in blocking buffer (1:5,000) was added to each well. In parallel, another membrane was

incubated with HRP-conjugated anti-rabbit Fc antibody (Jackson Laboratory, Bar Harbor, ME) diluted in blocking buffer. The immunoblot was developed using ECL (enhanced chemiluminescence) substrate (Pierce).

5. Affinity maturation of monoclonal antibodies

5.1. NNK randomization

Using degenerate primers, each of the residues in the complementarity determining region (CDR) of the antibody was randomized in the construction of the secondary library. For the secondary library, three or four consecutive residues in the CDR were randomized with an NNK nucleotide sequence as described previously (32). For LCDR1, LCDR2, and HCDR1, two secondary libraries were generated for each CDR; for LCDR3, HCDR2, and HCDR3, three secondary libraries were constructed. All secondary libraries were generated by overlap extension PCR, and the primer sequences are as follow: 5'-ACA CTT TAT GCT TCC GGC TC-3' and 5'-GGC CGG CCT GGC CAC TAG T-3' (33). To enrich higher affinity binders, these secondary libraries were subjected to four rounds of biopanning, and screening by phage ELISA were performed as described above.

5.2. Conversion of scFv to IgG_1

For production of full length IgG_1 monoclonal antibodies, variable region genes of the selected clones and each leader peptides were amplified by PCR. The primer sequences of V_L and V_H gene are as follow: V_L gene; 5'-ACC GGT GCC GCC ACC ATG GGC TGG TCC TGC ATC ATC CTG TTC CTG GTG GCC ACC GCC ACC GGC GAG CTC GTG CTG ACT CAG TCG CCC-3' and 5'-GGG ACC CAG CTG ACC GTC ACA GGC CGG ACC G-3', and V_H gene; 5'-GCT AGC CGC CAC CAT GGG CTG GTC CTG CAT CAT CCT GTT CCT GGT GGC CAC CGC CAC CGG CCA GTC GGT GGA GGA GTC CGG GGG T-3' and 5'-GGC ACC CTG GTC ACC GTC TCC TCG GCC TCC ACC AAG GGC CC-3'. The PCR reactions were carried out under the following conditions: 25 cycles of 30 sec at 94°C, 30 sec at 55°C, and 90 sec at 72°C, followed by a final extension for 10 min at 72°C. After then, each V_L and V_H genes were subcloned into human IgG_1 expression vector after digestion with *Age*I (5,000 U/mL) (NEB) and *Rsr*II (5,000 U/mL) (NEB) for V_L gene and *Nhe*I (50,000 U/mL) (NEB) and *Apa*I (50,000 U/mL) (NEB) for V_H gene as described previously (34). For overexpression of IgG_1, the expression vector was transfected into HEK293F cells using PEI and purified by affinity chromatography as described previously (28).

6. Epitope mapping of the antibody

Epitope mapping was performed using Ph.D.™-7 Phage Display Peptide Libraries (NEB) through a total of three rounds of biopanning on NPBR9 IgG_1 and NPBC20 IgG_1, immobilized on microtiter plates (Corning), as described previously (35). After every round of panning, individual phage plaques were grown on top agar plate and the output titration plate of the third round was used for bind to recombinant NT-proBNP-Fc fusion protein.

Specificity to NT-proBNP-Fc fusion protein with individual phage clones were confirmed by phage ELISA. Overnight culture of ER2738 (NEB) was diluted 1:100 in SB and 1 mL of diluted culture into deep well plate (Axygen), it used for phage amplification and characterization of each clone. Thirty-two clones of each antibody were infected into the diluted *E. coli* culture and incubated at 37°C with shaking for 5 h. After incubation, the phage cultures in deep well plate (Axygen) were centrifugated at 4000 rpm for 20 min, and then transfer the supernatant to a fresh tube. Amplified individual phages were used for phage ELISA and sequencing analysis for identification using sequencing primer (5'-CCCTCATAGTTAGCGTAACG-3', NEB) which encoding random peptides in the library.

7. Site-directed mutagenesis of NT-proBNP and determination of its reactivity

The genes encoding the recombinant NT-proBNP-Fc was used as the template for site-directed mutagenesis. Mutations were introduced by PCR and subcloned into the modified pCEP4 expression vector using *Sfi*I (Roche) as described above. The vectors encoding mutant proteins were transfected into HEK293F cells, and the recombinant proteins were purified by affinity chromatography as described above. Then, the reactivity of the mutant NT-proBNP-Fc fusion protein toward NPBR9 IgG_1 or NPBC20 IgG_1 was determined using a phage ELISA performed as described previously (26). The microtiter plates (Corning) were coated with each of the purified mutant NT-proBNP-Fc fusion proteins (4 μg/mL in PBS, 25 μL) overnight at 4°C. Then, the wells were blocked with 150 μL of 3% BSA in PBS (w/v) for 1 h at 37°C. The plates were washed with 150 μL of 0.05% PBST and incubated with each IgG_1 at 37°C for 90 min. Then, the plates were washed three times with 150 μL of 0.05% PBST. Subsequently, HRP-conjugated goat anti-human Fab antibody (Pierce) diluted in blocking buffer (1:5,000) was added to each well , and incubated for 1 h at 37°C. The plates were further washed three times with 150 μL of 0.05% PBST, and 50 μL of ABTS substrate solution (Pierce) was added to each well, followed by incubation for 30 min at 37°C. Finally, the OD was measured at 405 nm (Labsystems S.L.)

8. Overexpression and purification of a bispecific scFv-C_κ fusion protein

A gene sequence encoding the *Kpn*I restriction site, the leader sequence of the human Ig κ-chain followed by two *Sfi*I restriction sites, a sequence for a linker $(Gly\text{-}Gly\text{-}Gly\text{-}Ser)_3$ (36), the human Ig kappa chain C region (C_κ), a sequence for another linker $(Gly\text{-}Gly\text{-}Gly\text{-}Ser)_3$, and the restriction sites for *Age*I (NEB), *Not*I (NEB), and *Bam*HI, was chemically synthesized (Genscript, Piscataway, NJ) and cloned into the pCEP4 vector after digestion with the restriction enzymes *Kpn*I and *Bam*HI. Then, the anti-NT-proBNP scFv gene and anti-cotinine scFv (28) gene were cloned into the expression vector after being digested with either *Sfi*I or with *Age*I and *Not*I, respectively, as described previously (36). The vector was transfected into HEK293F cells and the recombinant protein was purified by affinity chromatography using the KappaSelect resin (GE Healthcare) following the manufacturer's instructions.

NPBR9 × anti-cotinine bispecific scFv-C_κ fusion protein (50 μg) was conjugated with HRP using peroxidase labeling kit (Dojindo, Rockville, MD) following the guidelines provided by the supplier. After HRP conjugation, HRP-conjugated NPBR9 × anti-cotinine bispecific scFv-C_κ fusion protein was stored at -20°C until use.

9. Real-time interaction analysis

The peptide mimotopes (pep 9-10; DVSSHRKGGGSC, pep 9-12; DIVGHRKGGGSC) were chemically synthesized and conjugated to BSA (Peptron Inc., Daejeon, Republic of Korea). Either the BSA-peptide conjugates or the recombinant NT-proBNP-Fc fusion protein was immobilized to a CM5 sensor chip (GE Healthcare) in 10 mM sodium acetate buffer (pH 5.0) using an amine coupling kit (GE Healthcare) at a flow rate of 5 μL/min. The NPBR9 × anti-cotinine bispecific scFv-C_κ fusion protein or NPBR9 scFv-C_κ fusion protein were dissolved in HBS-EP buffer (GE Healthcare) at concentrations ranging from 6.25 to 100 nM and injected over the chip for 3 min at a flow rate of 30 μL/min. After each binding assay, the chip was regenerated by washing with 10 mM glycine-HCl (pH 2.0) for 2 min at a flow rate of 30 μL/min. All the experiments were performed using the Biacore T200 instrument (GE Healthcare) at 25°C. The K_d values were calculated from kinetic association (k_{on}) and dissociation (k_{off}) constants, using the formula: $K_d = k_{off} / k_{on}$.

10. Competition assay using peptide mimotopes

10.1. Competition ELISA of using BSA-peptide conjugates

The microtiter plates (Corning) were coated with BSA-peptide conjugates (0.2 μg/mL in PBS, 25 μL) or the recombinant NT-proBNP-Fc fusion protein

(4 μg/mL in PBS, 25 μL) overnight at 4°C. The wells were blocked with 150 μL of 3% BSA in PBS (w/v) for 1 h at 37°C, and the plates were washed with 150 μL of 0.05% PBST. The recombinant NT-proBNP-Fc fusion protein was serially diluted 5-fold in 3% BSA in PBS (w/v) are pre-incubated with a 0.1 μg/mL solution of NPBR9 × anti-cotinine bispecific scFv-C_κ fusion protein or HRP-conjugated NPBR9 × anti-cotinine bispecific scFv-C_κ fusion protein in 3% BSA in PBS (w/v) for 15 min at room temperature. The mixture was add to each well and incubated for 2 h at 37°C. After washing three times with 150 μL of 0.05% PBST, HRP-conjugated cotinine (1:1,000) were added to each well (28) that were incubated with NPBR9 × anti-cotinine bispecific scFv-C_κ fusion protein previously. The plates were incubated for 1 h at 37 °C followed by washing three times with 150 μL of 0.05% PBST. Fifty microliters of 3,3',5,5'-tetramethylbenzidine (TMB) substrate solution (GenDEPOT, Texas, US) were added to each well. After incubation, 50 μL of 2 M H_2SO_4 was added to stop the reaction. Subsequently, the OD was measured at 450 nm (Labsystems S.L.).

10.2. Competition ELISA of unconjugated free peptide mimotopes

The Sulfhydryl-BIND™ well plates (Corning) were coated peptide mimotopes through cysteine residue in C-terminal end (100 μg/mL in PBS (pH 6.5) with 1 mM EDTA, 50 μL) at room temperature for 2 h. The plates were washed with 200 μL of PBS and the wells were sequentially blocked

with 200 μL of 100 mM cysteine and 3% BSA in PBS (w/v) at room temperature for 1 h. Then the plates were washed three times with 200 μL of PBS. The recombinant NT-proBNP-Fc fusion protein was serially diluted 10-fold in PBS and pre-incubated with a 5 μg/mL solution of NPBR9 × anti-cotinine bispecific scFv-C_κ fusion protein in PBS as described above. The mixture was added to each well and incubated at room temperature for 2h. After washing with PBS, HRP-conjugated cotinine (1:1,000) were added to each well. The plates were incubated for 1 h at room temperature followed by washing three times. One hundred microliters of TMB substrate solution (GenDEPOT) were added to each well. After incubation, same volume of 2M H_2SO_4 was added to stop the reaction. Subsequently, the OD was measured at 450 nm (Labsystems S.L.).

10.3. Real-time competition analysis using BSA-peptide conjugates

BSA-peptide conjugates and the recombinant NT-proBNP-Fc fusion protein were immobilized to a CM5 sensor chip (GE Healthcare) as described above. The recombinant NT-proBNP-Fc fusion protein was serially diluted 5-fold in HBS-EP buffer (GE Healthcare) at concentration ranging from 0.08 to 50 nM and injected over the chip as described above. Each of these solutions was pre-incubated with 50 nM of NPBR9 × anti-cotinine bispecific scFv-C_κ fusion protein in HBS-EP buffer (GE Healthcare) at room temperature and

injected over the chip for 3 min at a flow rate of 30 µL/min. After each competition analysis, the chip was regenerated as described above.

11. Sandwich ELISA

The microtiter plates (Corning) were coated with monoclonal mouse anti-human NT-proBNP; $15F11_{13-27}$ (Hytest, Finland) (8 µg/mL in PBS, 25 µL) overnight at 4°C. The wells were blocked with 150 µL of 3% BSA in PBS (w/v) for 1 h at 37°C. The plates were washed with 150 µL of 0.05% PBST and incubated with glycosylated or de-glycosylated recombinant NT-proBNP-Fc fusion protein serially diluted 3-fold in 3% BSA in PBS (w/v) for 1 h at 37°C. The plates were washed repeatedly as described above; next, a 2 µg/mL solution of each anti-NT-proBNP × anti-cotinine bispecific scFv-C_κ fusion protein was added to each well, and the plates were incubated for 2 h at 37°C. After washing three times with 150 µL of 0.05% PBST, HRP-conjugated cotinine (1:250) was added to each well. The plates were incubated for 1 h at 37°C and washed three times with 150 µL of 0.05% PBST. Following this, 50 µL of TMB substrate was added to each well followed by incubation, and 50 µL of 2M H_2SO_4 was added to stop the reaction. The OD was the measured at 450 nm (Labsystems S.L.).

In a parallel experiment, the same procedure was performed up to the blocking step. Subsequently, the plates were incubated with 2 µg/mL of NPBR9 IgG_1 or HRP-conjugated $24E11_{61-76}$ (Hytest). The plates were washed

three times with 150 μL of 0.05% PBST, and HRP-conjugated anti-human Fc antibody (1:5,000) was added to each well. After washing the plates, each plate was incubated with 50 μL of ABTS substrate for 30 min at 37°C. The OD was then measured at 405 nm (Labsystems S.L.).

RESULTS

1. Overexpression of glycosylated NT-proBNP as a Fc fusion protein

The gene encoding human NT-proBNP gene was amplified by PCR from the cDNA which prepared from HEK (human embryonic kidney) cells **(Fig. 6)**. To develop the human NT-proBNP-Fc fusion protein, the expression vector encoding the human NT-proBNP-Fc gene was transfected into HEK293F cells. The recombinant NT-proBNP-Fc fusion protein was purified from the culture supernatant by protein A column chromatography. A broad protein band with a molecular weight of 50 kDa was observed upon SDS-polyacrylamide gel electrophoresis after Coomassie staining **(Fig. 7A)**. The expected molecular weight of the fusion protein was 39 kDa, and we confirmed its identity in an immunoblot assay using the anti-rabbit Fc antibody, which reacted well with the protein band **(Fig. 7B)**. To further confirm its identity as NT-proBNP, the protein was eluted from the gel and analyzed using mass spectrometry. The mass spectrometry data confirmed that the band represent the recombinant NT-proBNP-Fc fusion protein (data not shown).

Because it has previously been reported that NT-proBNP is heavily *O*-glycosylated, we determined whether the higher molecular weight and the heterogeneity of the band are due to glycosylation. The purified recombinant

NT-proBNP-Fc fusion protein was treated with of *O*-glycosidase and neuraminidase to remove *O*-linked carbohydrate moieties, and then subjected to SDS-polyacrylamide gel electrophoresis and immunoblot analysis. As in Figure 5A, the heterogeneity and average molecular weight of the scFv-Fc fusion protein, which has only one *N*-glycosylation site in the Fc region, was used as a negative control. Bovine fetuin was used as a positive control and showed decreased molecular weight after treatment. These results indicate that the recombinant NT-proBNP-Fc fusion protein is heavily *O*-glycosylated.

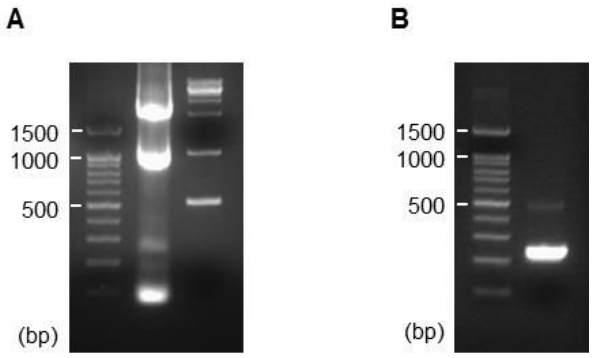


Figure 6. Amplification of the human NT-proBNP gene. (A) mRNA preparation from HEK and (B) amplification of the human NT-proBNP gene by PCR. Each RNA or DNA were loaded and run on an agarose gel.

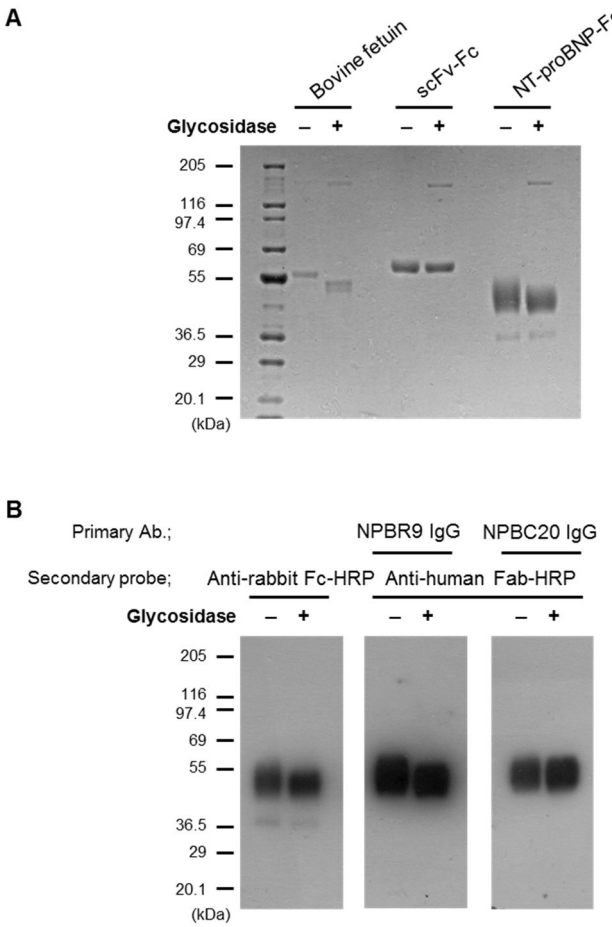


Figure 7. Expression and de-glycosylation of the recombinant NT-proBNP-Fc fusion protein. (A) The recombinant NT-proBNP-Fc was expressed in HEK293F cells and purified by protein A column chromatography. The purified protein, bovine fetuin (*O*-glycosylated protein control), and an scFv-Fc protein (non *O*-glycosylated protein control) were treated with glycosidase and subjected to SDS-polyacrylamide gel electrophoresis and Coomassie staining along with the non-treated protein. (B) The untreated or treated recombinant NT-proBNP-Fc fusion proteins

separated on the gel were transferred to a nitrocellulose membrane and probed with the NPBR9 antibody or the NPBC20 antibody, followed by an anti-human Fc antibody conjugated with HRP. The membrane was also probed with anti-rabbit Fc antibody conjugated with HRP to visualize the recombinant NT-proBNP-Fc fusion protein.

2. Selection of antibodies to NT-proBNP and development of enzyme immunoassay

After immunization of rabbits with the recombinant NT-proBNP-Fc fusion protein, titration serum antibody was determined by enzyme immunoassay **(Fig. 8)**. Total RNA was prepared from bone marrow and spleen, and the quality of RNA is assessed by electrophoresis with agarose gel **(Fig. 9A)**. The genes encoding V_H and V_L of rabbit immunoglobulin was amplified from the cDNA library by PCR **(Fig. 9B and C)**. Finally, rabbit immune scFv library was constructed using overlap extension PCR **(Fig. 9D)**.

The genes encoding the NT-proBNP scFv was cloned into a phagemid vector (pComb3X) for displaying anti-NT-proBNP scFv on the surface of M13 phage. The phagemid library was transformed into *E. coli* cells and the scFv-displaying phages were rescued. A phage display library of combinatorial scFvs was constructed, with a complexity of 2.2×10^9. A naïve chicken scFv library, with a complexity of 1.3×10^9, was also subjected to the biopanning process for the selection of binders to NT-proBNP.

For biopanning, recombinant NT-proBNP overexpressed in *E. coli* was conjugated to paramagnetic beads using the epoxy groups on the beads. After biopanning, the phage clones from the output titer plates in the last round were rescued and subjected to a phage enzyme immunoassay using microtiter plates coated with the recombinant NT-proBNP-Fc fusion protein prepared in either prokaryotic or mammalian expression systems. Fourteen antibody

clones from the rabbit immune library and two antibody clones from the chicken naïve library showed reactivity to both antigens and were selected for further study.

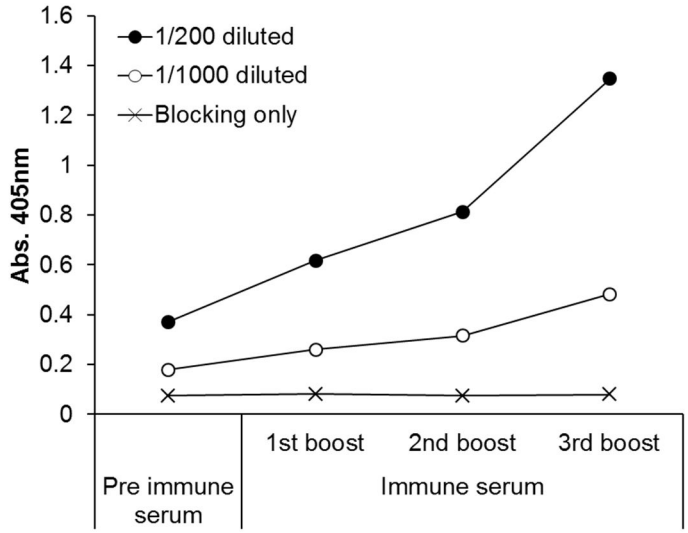


Figure 8. Titration of serum antibodies. The immune titers of anti-NT-proBNP antibodies in rabbit serum were measured by ELISA.

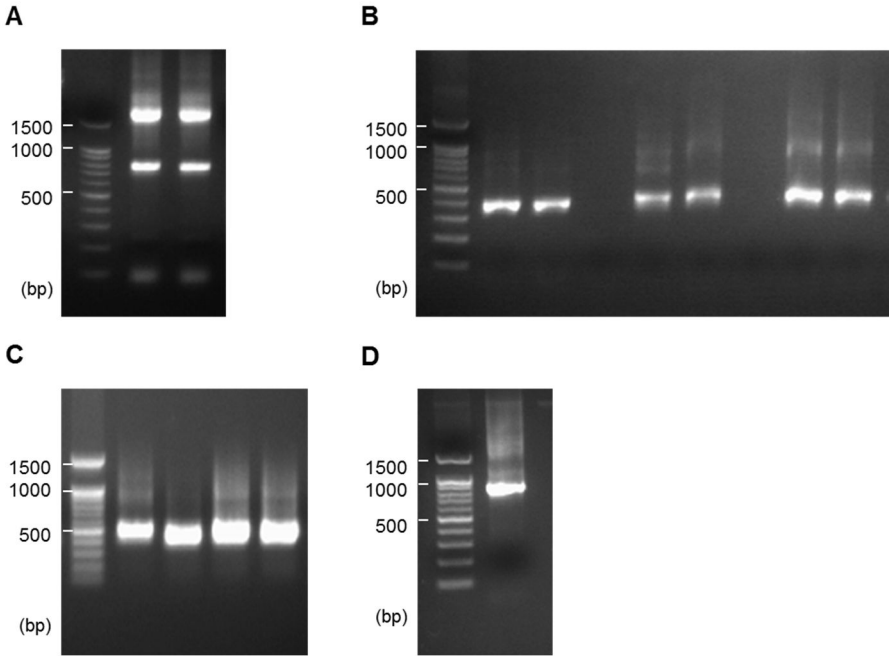


Figure 9. Construction of scFv libraries by overlap PCR. (A) Total RNA preparation from rabbit spleen and bone marrow. (B) Amplification of rabbit V_L fragments and (C) V_H fragments and (D) Overlap extension PCR fragments were loaded and run on an agarose gel.

3. Reactivity of antibodies to recombinant NT-proBNP-Fc fusion protein

To select an antibody clone reactive to glycosylated NT-proBNP, we performed immunoblot assays using both glycosylated and de-glycosylated recombinant NT-proBNP-Fc fusion protein. Those antibody clones whose reactivities were hindered by glycosylation, such as NPBC20, showed similar immunoblot bands in the lanes loaded with either untreated or de-glycosylated recombinant NT-proBNP-Fc fusion protein **(Fig. 7B, right)**. In addition, antibody clones whose reactivities were not affected by glycosylation also showed reactivity to the heavily glycosylated and higher molecular weight recombinant NT-proBNP-Fc fusion protein in the lane loaded with untreated recombinant NT-proBNP-Fc fusion protein **(Fig. 7B, left)**. An anti-human Fc antibody, a monoclonal antibody not-affected by *O*-glycosylation, was used as a positive control. In this analysis, the NPBR1 scFv-Fc fusion protein, a rabbit antibody clone, showed an immunoblot pattern similar to that of anti-Fc antibody, suggesting that its reactivity is not affected by *O*-glycosylation. The NPBR1 antibody clone was subjected to a process of affinity maturation. A higher affinity, *O*-glycosylation-independent daughter antibody clone, (NBBR9) **(Fig. 7B, middle)**, was selected for further study. NPBC20 was selected as a control antibody that was affected by *O*-glycosylation.

4. Determination of epitopes

To define the epitopes of NPBR9 IgG_1 and NPBC20 IgG_1, we employed a phage-displayed random heptamer peptide library. Three rounds of biopanning were performed using microtiter plates coated with NPBR9 IgG_1 and NPBC20 IgG_1. The phage clones were rescued from the output titer plate of the third round and subjected to a phage enzyme immunoassay. Phagemid DNA was prepared from nine clones reactive to NPBR9 IgG_1 and eleven clones reactive to NPBC20 IgG_1, and subjected to nucleotide sequence analysis. Among the NPBR9 clones, four, two, two, one, and one clones had HRK, HSK, HQK, HWK, HKK sequences, respectively; these sequences were identical or very much homologous to $H_{64}R_{65}K_{66}$ in NT-proBNP **(Fig. 10A)**. All the NPBC20 clones had VWK sequences, which were identical to $V_{50}W_{51}K_{52}$ in NT-proBNP **(Fig. 10B)**.

To further confirm the epitopes, recombinant NT-proBNP-Fc fusion protein mutants, bearing alanine or leucine substitutions at single amino acid residues in the HRK or VWK motifs or their flanking residues, were prepared and subjected to an enzyme immunoassay **(Fig. 11 and Fig. 12)**. The recombinant NT-proBNP-Fc fusion protein mutants were individually coated onto microtiter plates by the addition of solution at the same concentration. After blocking, NPBR9 IgG_1 or NPBC20 IgG_1 was added to each well. The amount of IgG_1 bound to the plate was determined using anti-human Fab antibody conjugated with HRP and ABTS substrate. In case of NPBR9 IgG_1,

substitution of G_{63} or K_{66} in NT-proBNP completely abolished its reactivity to the antibody, whereas in the case of NPBC20 IgG_1,these mutants showed similar reactivity to that of wild type. Substitution of H_{64} reduced its reactivity to NPBR9 IgG_1 compared to NPBC20 IgG_1, but the effect was minimal. The substitution of R_{62}, R_{65}, M_{67}, and V_{68} in NT-proBNP did not affect the reactivity of NBPR9 IgG_1 significantly. From this observation, we conclude that the epitope of NPBR9 IgG_1 includes G_{63}, H_{64}, R_{65}, and K_{66} **(Fig. 11)**.

In parallel, the specific reactivity of NPBC20 IgG_1 depended on G_{49}, V_{50}, K_{52}, S_{53}, R_{54}, and E_{55} in NT-proBNP, and the substitution of W_{51} in NT-proBNP completely abolished the reactivity to both antibodies. On the other hand, the substitution of flanking residues; P_{48} and V_{56} was permissive. Therefore the epitope of NBPC20 IgG_1 could be localized to G_{49}–E_{55} (fig. 12).

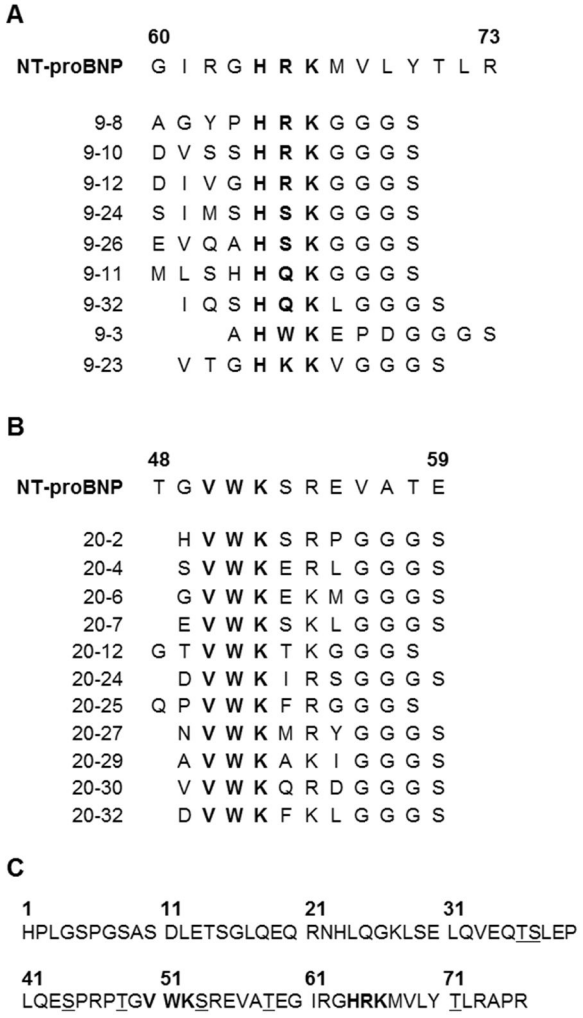


Figure 10. Alignment of peptide sequences. Peptide sequences of phage clones reactive to (A) NPBR9 antibody and (B) NPBC20 antibody, and (C) the peptide sequence of NT-proBNP. Consensus sequences are written in bold and *O*-linked glycosylation sites are marked by underline.

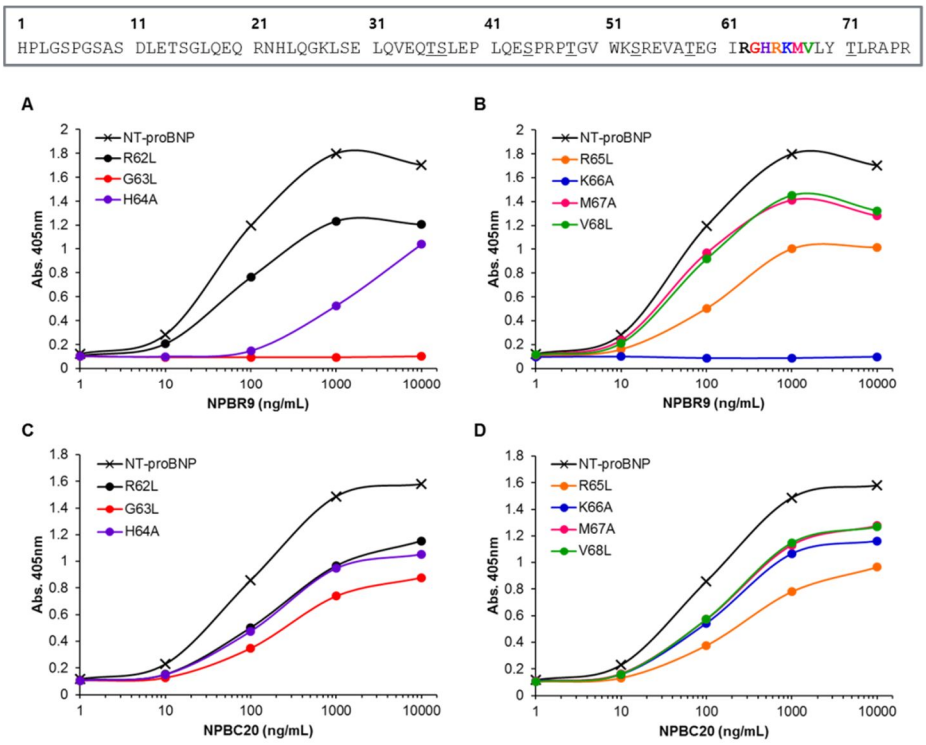


Figure 11. Site-directed mutagenesis of NT-proBNP-Fc fusion protein to determine the epitope of NPBR9. Single-residue mutants of NT-proBNP-Fc fusion protein were expressed in HEK293F cells. The mutant proteins were coated onto the microtiter plate. After blocking, **(A, B)** NPBR9 or **(C, D)** NPBC20 antibodies were incubated as the primary antibody. The amount of bound antibody was determined using anti-human Fc antibody conjugated to HRP.

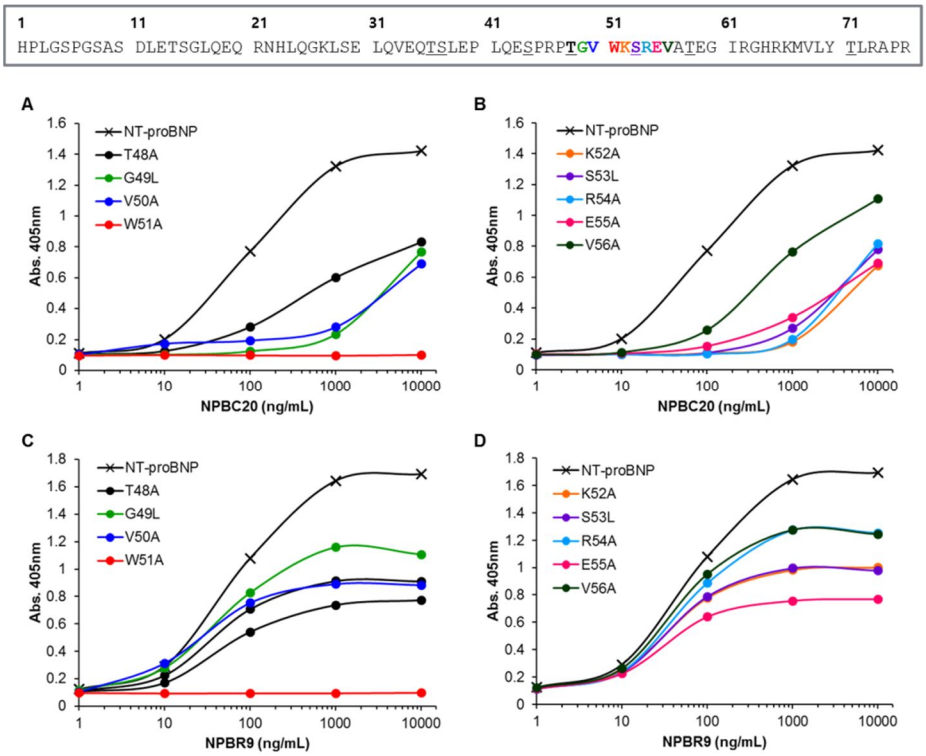


Figure 12. Site-directed mutagenesis of NT-proBNP-Fc fusion protein to determine the epitope of NPBC20. Single-residue mutants of NT-proBNP-Fc fusion protein were expressed in HEK293F cells. The mutant proteins were coated onto the microtiter plate. After blocking, **(A, B)** NPBC20 or **(C, D)** NPBR9 antibodies were incubated as the primary antibody. The amount of bound antibody was determined using anti-human Fc antibody conjugated to HRP.

5. Real-time interaction analysis of NPBR9 antibodies

To determine the affinity between NPBR9 antibody and peptide mimotopes, 9-10 and 9-12 peptides were chemically synthesized with the –GGGSC linker at C-terminal end and conjugated to BSA **(Fig. 13A)**. In real-time interaction analysis the BSA-peptide conjugates or the recombinant NT-proBNP-Fc fusion protein were chemically cross-linked to a CM5 sensor chip. In case of NPBR9 × anti-cotinine bispecific scFv-C_κ fusion protein, it was ordinarily dimerization through anti-cotinine scFv **(Fig. 13B)**. We could not exactly measurement of antibody affinity to BSA-peptide conjugates or fusion protein, because it was work as a bivalent antibody. Therefore we used NPBR9 scFv-C_κ fusion protein as a control for NPBR9 antibody. The k_{on}, k_{off} and K_d constant of NPBR9 antibody to BSA-peptide conjugates and the fusion protein was successfully determined and are summarized in Table 1. The affinity of scFv-C_κ fusion protein to the recombinant NT-proBNP-Fc fusion protein was quite similar to that of the NPBR9 × anti-cotinine bispecific scFv-C_κ fusion protein. However in the case of BSA-peptide conjugates, the affinity of the NPBR9 × anti-cotinine bispecific scFv-C_κ fusion protein was much higher than that of the scFv-C_κ fusion protein, which may be due to the bivalent nature of the former.

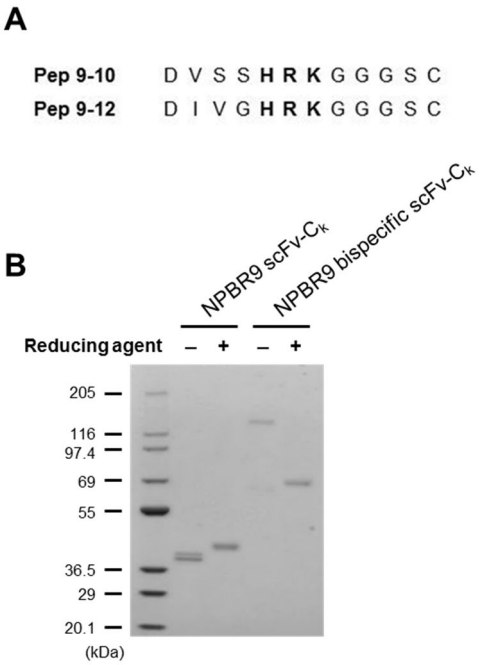


Figure 13. Peptide sequences and dimerization of the NPBR9 antibody. (A) Two peptide mimotopes were synthesized with –GGGSC linker at the C-terminal end. These peptide sequences obtained from epitope mapping of NPBR9 IgG_1. (B) NPBR9 scFv-C_κ fusion protein or NPBR9 × anti-cotinine bispecific scFv-C_κ fusion protein was treated with reducing agent or untreated (left), and subjected to SDS-polyacrylamide gel electrophoresis and Coomassie blue staining. The NPBR9 scFv-C_κ fusion protein was used as a control.

Table 4. Real-time interaction analysis of NPBR9 antibody

Analyte	Ligand	k_a (1/Ms)	k_d (1/s)	K_D (M)
NPBR9 bispecific scFv-C_k	Pep 9-10/BSA	1.935E+5	2.486E-4	1.287E-9
	Pep 9-12/BSA	1.232E+4	3.548E-5	2.881E-9
	NT-proBNP-Fc	2.524E+4	4.272E-4	1.692E-8
NPBR9 scFv-C_k	Pep 9-10/BSA	7.499E+3	3.291E-4	4.389E-8
	Pep 9-12/BSA	9.717E+3	2.392E-4	2.462E-8
	NT-proBNP-Fc	4.605E+4	1.487E-3	3.229E-8

6. Competition assays with peptide mimotopes

To further confirm that the peptides represent the epitope, a competition assay was developed. BSA-peptide conjugates were coated onto a microtiter plate. The recombinant NT-proBNP-Fc fusion protein was serially diluted, pre-incubated with NPBR9 × anti-cotinine bispecific scFv-C_κ fusion protein, and added to each well. Next, HRP-conjugated cotinine and TMB substrate were sequentially added. In this competition assay the binding of the antibody to peptides was decreased at concentrations of 0.3 ng/mL or higher in a dose-dependent manner **(Fig. 14A)**. In a parallel experiment using the recombinant NT-proBNP-Fc fusion protein-coated plate, the addition of the recombinant NT-proBNP-Fc fusion protein to the soluble protein decreased antibody binding to the plate in a similar dose-dependent manner **(Fig. 14C)**. However when we incubated with HRP-conjugated NPBR9 × anti-cotinine bispecific scFv-C_κ fusion protein, this competitive assay system could not sensitively detection to NT-proBNP **(Fig. 14B and D)**. It may be that lots of HRP molecules were conjugated with NPBR9 × anti-cotinine bispecific scFv-C_κ fusion protein, so it was not suitable for detection of low amounts of NT-proBNP. On the other hand, the addition of irrelevant Fc fusion protein did not affect the binding of the NPBR9 × anti-cotinine bispecific scFv-C_κ fusion protein either to BSA-peptide conjugates or the recombinant NT-proBNP-Fc fusion protein coated on the plate (data not shown).

In addition, we performed another competition assay with the free peptide

mimotopes. The free peptides were coated Sulfhydryl BIND™ well plate. The recombinant NT-proBNP-Fc fusion protein was serially diluted, pre-incubated with NPBR9 × anti-cotinine bispecific scFv-C_κ fusion protein, as described above, and added to each well. The free peptides were also competed with soluble recombinant NT-proBNP-Fc fusion protein like as BSA-conjugated peptides. However this assay system could work on over 100 ng/mL of NT-proBNP **(Fig. 14E)**.

The competition assay was also performed using the surface plasmon resonance (SPR) format. BSA-peptide conjugates were immobilized using amine coupling chemistry to a CM5 sensor chip. The binding of the antibody to the chip was decreased by the addition of a fusion protein in a dose-dependent manner at a concentration of 0.4 nM or higher **(Fig. 15).** A similar pattern of decrease in binding was observed in a parallel experiment using a fusion protein conjugated chip.

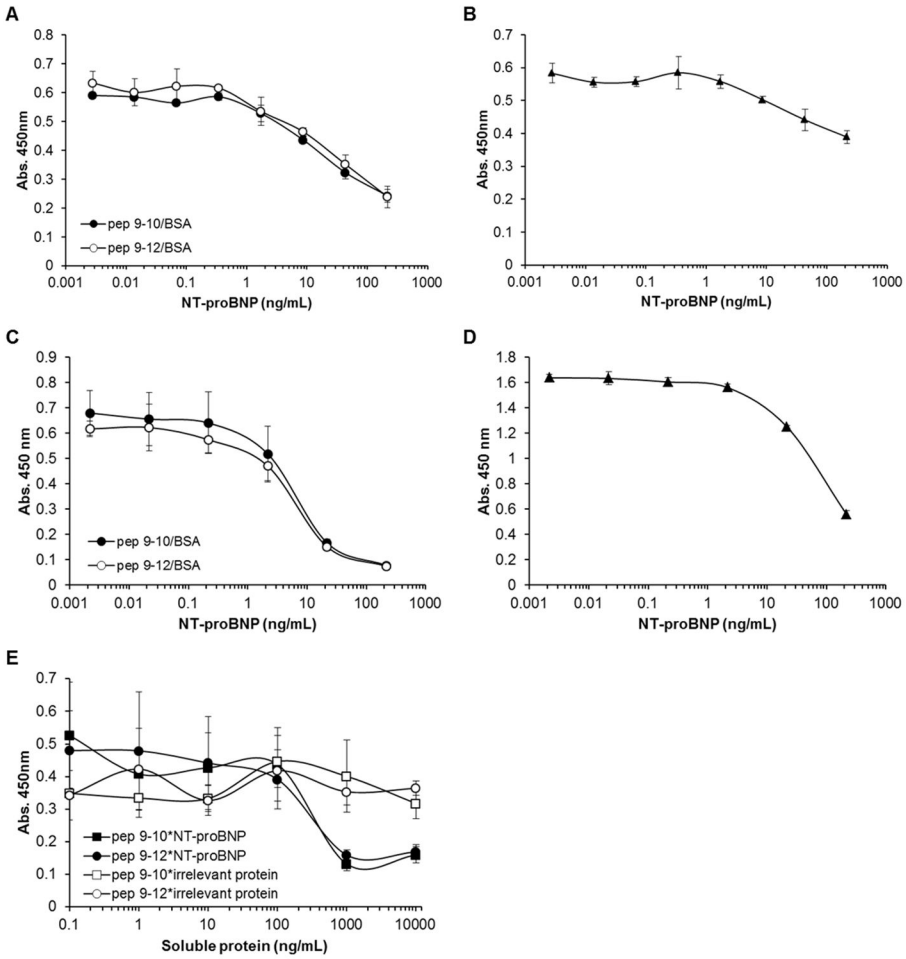


Figure 14. Competition ELISA using peptide mimotopes. The microtiter plate was coated with either (A, C) BSA-peptide conjugates or (B, D) recombinant NT-proBNP-Fc fusion protein. (A, B) Serially diluted recombinant NT-proBNP-Fc fusion protein was pre-incubated with NPBR9 × anti-cotinine bispecific scFv-C_κ fusion protein, and the mixture was added into each well. The amount of bound NPBR9 antibody was determined using HRP-conjugated cotinine. (C, D) In parallel experiments, serially diluted

recombinant NT-proBNP-Fc fusion protein was pre-incubated with HRP-conjugated NPBR9 × anti-cotinine bispecific scFv-C_{κ} fusion protein. (E) Competition ELISA with free peptide mimotope and recombinant NT-proBNP-Fc fusion protein (●, ■). Irrelevant protein (○, □) was added as a control for recombinant NT-proBNP-Fc fusion protein.

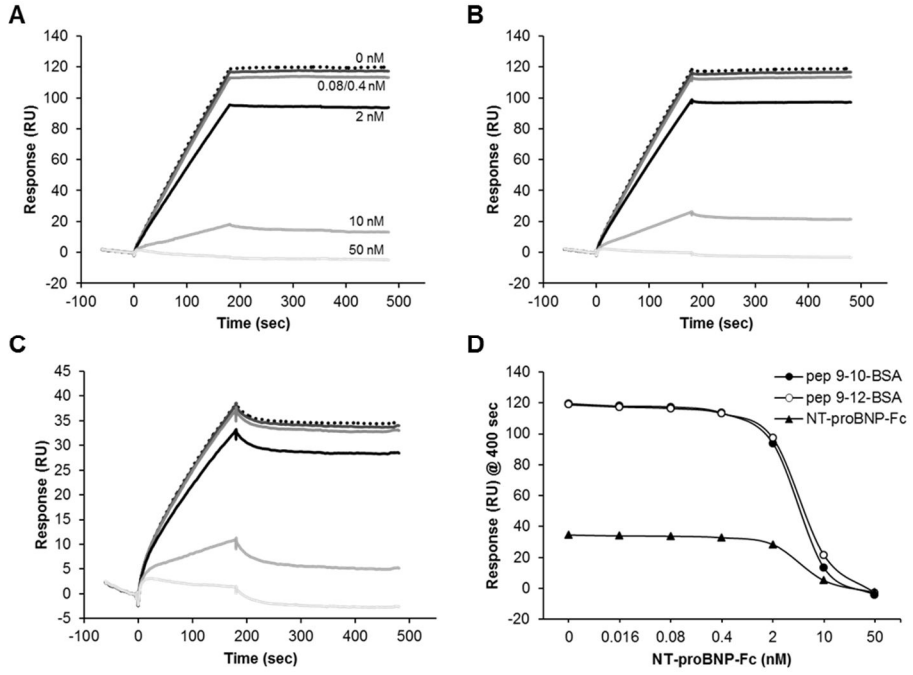


Figure 15. Competition assays using BSA-peptide conjugates in real-time interaction analysis. Three different ligands, including (A) pep 9-10/BSA, (B) pep 9-12/BSA, and (C) the recombinant NT-proBNP-Fc fusion protein were immobilized on a CM5 sensor chip. Serially diluted recombinant NT-proBNP-Fc fusion protein was pre-incubated with the NPBR9 × anti-cotinine bispecific scFv-C_κ fusion protein, and the mixture was injected over the chip. (D) Comparison of the results of each competition assay at 400 sec in SPR analysis.

7. Sandwich ELISA using NPBR9 antibody

To further confirm the binding of NPBR9 was not affected by the *O*-glycosylation of NT-proBNP, we performed a sandwich enzyme immunoassay. The $15F11_{13\text{-}27}$ antibody, reactive to an epitope encompassing the 13^{th} to 27^{th} residue of NT-proBNP, was coated onto the plate. Subsequently, either untreated or de-glycosylated recombinant NT-proBNP-Fc fusion protein was serially diluted 3-fold and added to each well. Following this, the binding of the NPBR9 or NPBC20 × anti-cotinine bispecific scFv-C_κ fusion proteins was determined using HRP-conjugated cotinine. The NPBR9 antibody exhibited similar binding to untreated as well as de-glycosylated recombinant NT-proBNP-Fc fusion protein, whereas the binding of NPBC20 × anti-cotinine bispecific scFv-C_κ fusion protein was significantly increased upon de-glycosylation **(Fig. 16A)**. We compared the binding characteristics of NPBR9 IgG_1 with the $23E11_{61\text{-}76}$ antibody reactive to an epitope encompassing residues 61–76, including the glycosylated Thr_{71} residue. As expected, the binding of NPBR9 IgG_1 was not significantly affected by the *O*-glycosylation status of the recombinant NT-proBNP-Fc fusion protein whereas the binding of the $23E11_{61\text{-}76}$ antibody was dramatically increased on de-glycosylation **(Fig. 16B)**.

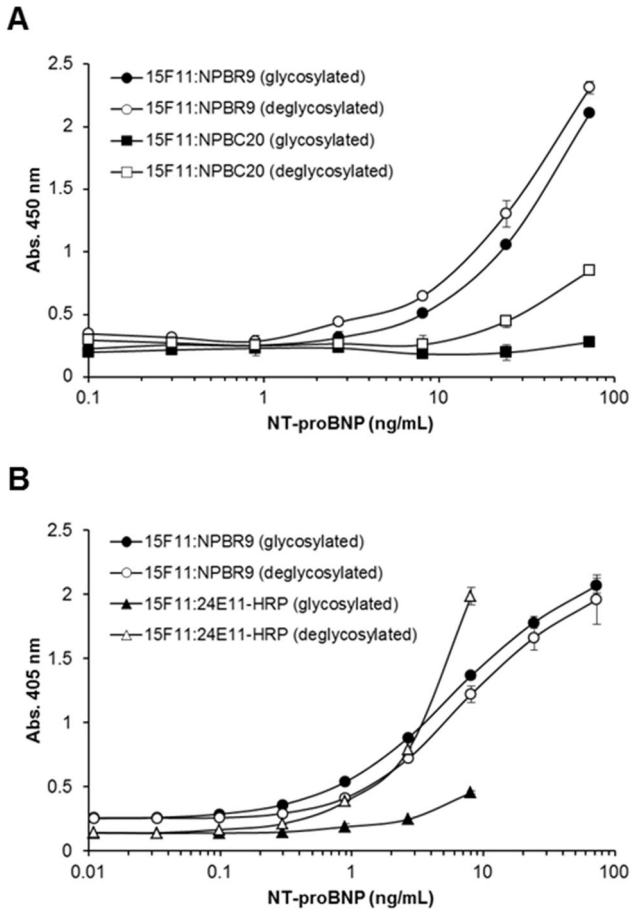


Figure 16. Sandwich ELISA using NPBR9 antibody. The $15F11_{13\text{-}27}$ antibody reactive to residues 13–27 of NT-proBNP, was coated onto a microtiter plate. Glycosidase treated (○, □) or untreated (●, ■) recombinant NT-proBNP-Fc fusion protein was serially diluted and added to each well. **(A)** Nest, wells were incubated with NPBR9 × anti-cotinine bispecific scFv-C_κ fusion protein or NPBC20 × anti-cotinine bispecific scFv-C_κ fusion protein and subsequently probed with HRP-conjugated cotinine, followed by HRP

substrate. **(B)** Alternatively, the plate was incubated with either NPBR9 IgG_1 and anti-human Fc antibody conjugated to HRP in succession, or an anti-NT-proBNP $24E11_{61\text{-}76}$ antibody conjugated to HRP, followed by HRP substrate.

Discussion

Antibody recognition of NT-proBNP is influenced by both the glycosylation pattern(15) and its fragmentation in blood. NT-proBNP in human blood is reported to be *O*-glycosylated at seven residues (Thr_{36}, Ser_{37}, Ser_{44}, Thr_{48}, Ser_{53}, Thr_{58} and Thr_{71}) (12). Five of these residues are found to be always glycosylated in all NT-proBNPs and two residues (Thr_{36} and Thr_{58}) are glycosylated only in a fraction of NT-proBNPs (12). This glycosylation pattern affects the recognition of the NT-proBNP molecule by antibodies (13). When antibodies are raised by immunization with non-glycosylated recombinant NT-proBNP, antibodies reactive to epitopes consisting of residues 28–45, 31–39, 34–39, and 46–56 showed drastically reduced reactivity to glycosylated NT-proBNP extracted from pooled plasma of heart failure patients compared with the de-glycosylated form.

Recently, it was reported that immunoreactive NT-proBNP is present as multiple N- and C- terminally truncated fragments, based on a study in which NT-proBNP molecules were immunoprecipitated from the pooled plasma of four heart failure patients using an antibody reactive to epitope 13–20, digested with trypsin and subjected to liquid chromatography (LC)-electrospray ionization-tandem mass spectrometry (MS/MS) (22). In the spectrum several peptides were identified; these were semi-tryptic peptides, in which one end of the peptide was not the result of cleavage at consensus

trypsin recognition sites. The physiological proteolysis sites were expected to be between the Pro_2–Leu_3, Leu_3–Gly_4, Pro_6–Gly_7, and Pro_{75}–Arg_{76} residue pairs. Consistent with these results, AlphaLISA immunoassays demonstrated that antibodies targeting the extreme N- or C- termini measured a low apparent concentration of circulating NT-proBNP (22). The apparent circulating NT-proBNP concentration was higher when antibodies targeting non-glycosylated and non-terminal epitopes were used in the immunoassay. The major limitation of this study was that NT-proBNP molecules physiologically fragmented beyond the 30th residue could not be enriched by the immune-precipitation process.

Based on the observation that circulating NT-proBNP is heavily and heterogeneously *O*-glycosylated and fragmented extensively from both ends, it is clear that the antibody binding site is important in the development of the enzyme immunoassay. There are several clinically available sandwich immunoassays. One assay employs polyclonal antibodies against epitopes of NT-proBNP$_{1-21}$ and NT-proBNP$_{39-50}$ (23). Another polyclonal antibody pair that recognizes NT-proBNP$_{8-29}$ and NT-proBNP$_{31-57}$ is used in another assay kit (24). Monoclonal antibody pairs recognizing NT-proBNP$_{13-27}$ and NT-proBNP$_{61-76}$ or NT-proBNP$_{1-21}$ and NT-proBNP$_{61-76}$ are also employed (13). The reactivity of antibodies directed against NT-proBNP$_{39-50}$ and NT-proBNP$_{31-57}$ could be affected by the glycosylation pattern, those directed against NT-proBNP$_{1-21}$ and NT-proBNP$_{8-29}$ by physiological fragmentation and those against NT-proBNP$_{13-27}$ and NT-proBNP$_{61-76}$ by both modifications.

Compared to these monoclonal antibodies, the epitope of NPBR9 was more precisely defined to be $G_{63}H_{64}R_{65}K_{66}$ by mimetic peptide screening **(Fig. 10)**, site-directed mutagenesis **(Fig. 11 and Fig. 12)**, and competition assay with peptide mimotope **(Fig. 14A)**. As the nearest *O*-glycosylation residues are Thr_{58} and Thr_{71}, four amino acid residues intervene between the epitope and those residues in both directions. As there are only six, three, and four amino acid residue gaps between the two *O*-glycosylation residues in Ser_{37}–Ser_{44}, Ser_{44}–Thr_{48}, and Thr_{48}–Ser_{53}, respectively, this epitope provides the longest gaps between the two *O*-glycosylation residues. Furthermore, only four amino acid residues are present between *O*-glycosylated Thr_{71} and Pro_{75} after possible physiological proteolysis. Based on these facts, the epitope $G_{63}H_{64}R_{65}K_{66}$ has the longest distances from the *O*-glycosylation residues. There has been no report that two antibodies for a sandwich immunoassay can bind to the 28-amino acid residue-long region between Gly_7 and *O*-glycosylated Thr_{36} in physiologically digested NT-proBNP, therefore, one antibody reactive to this 28-amino acid residue-long region and the other antibody binding to $G_{63}H_{64}R_{65}K_{66}$ is an ideal pair for an sandwich immunoassay detecting the greatest amount of *O*-glycosylated and physiologically digested NT-proBNP.

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Abstract in Korean

나트륨이뇨 펩타이드의 N-말단 전구 물질인 NT-proBNP 는 울혈성 심부전증을 진단하기 위한 중요한 바이오마커이다. 비록 NT-proBNP 의 생물학적 기능은 잘 알려져 있지 않으나, 이것은 바이오마커로서의 많은 이점을 가지고 있다. NT-proBNP 는 여러 개의 *O*-글리코실화 사이트를 가지고 있으며, 혈액 내에 존재하는 NT-proBNP 또한 글리코실화 되어 있다고 알려져 있다. 따라서 글리코실화-비의존적 결합을 하는 항체를 개발하는 것은 매우 어려운 일이다. 게다가 생리학적인 단백질 가수분해에 의해 N- 또는 C-말단 부분이 잘려진 NT-proBNP 단편이 관찰되기도 하였다. 재조합 NT-proBNP-Fc 융합 단백질은 혈액 내에 존재하는 NT-proBNP 와 마찬가지로 글리코실화 되어 있다. 항-NT-proBNP 단클론 항체인 NPBR9 은 토끼 면역 라이브러리로부터 생성되었으며, 이것은 이중특이성 scFv-C_{κ} 융합 항체 및 IgG_1 항체 형태로 개조되었다. 이 항체의 항원에 대한 결합 부위는 무작위적 펩타이드 라이브러리를 이용한 파지 디스플레이 및 위치 선택적 돌연변이 유발 실험을 통해 특징 분석을 하였다. NPBR9 항체의 에피톱은 NT-proBNP 의 C-말단 인근에 위치하고 있으나, 단백질 가수분해에 취약한 C-말단의 가장 끝 부분은 아닌 것으로 밝혀졌다. 이 에피톱은 "$H_{64}R_{65}K_{66}$"의

3 개의 아미노산으로 구성된 공통 서열을 가지고 있다. 또한 우리는 NT-proBNP 의 G_{63} 또는 K_{66} 잔기가 치환된 경우, 항체에 대한 반응성이 완전히 사라지는 것을 알게 되었다. 뿐만 아니라, C-말단에 –GGGSC 링커를 연결한 펩타이드 미모톱을 합성하였고, 이것은 경쟁적 효소 면역측정법을 통해 재조합 NT-proBNP-Fc 융합 단백질과 경쟁을 하는 것을 확인하였다. 우리는 이 연구를 통해 글리코실화 및 양쪽 말단의 절단에 거의 영향을 받지 않는 에피톱에 반응하는 항체를 개발하고자 하였다. 다음에는 이 항체와 에피톱을 모방한 펩타이드를 이용하여 경쟁적 효소 면역측정법을 수행하였다. 그리고 샌드위치 효소 면역 측정법을 통해 NPBR9 항체가 글리코실화 된 재조합 NT-proBNP 및 탈당화 된 NT-proBNP 모두에 비슷한 반응성을 가지고 있다는 것을 확인하였다. 그러나 우리가 개발한 경쟁적 효소 면역측정법은 매우 낮은 농도의 NT-proBNP 의 민감한 검출을 하기에는 충분하지 않았다. 이미 선행 연구를 통해 NPBR9 항체가 글리코실화 및 양쪽 말단의 절단에 영향을 받지 않는 것을 알았기 때문에, 이 항체를 샌드위치 효소 면역측정법의 검출항체로 적용해 보았다. 상업적으로 이용되는 효소 면역측정법으로는 글리코실화 되었거나 단편으로 존재하는 NT-proBNP 의 검출이 어려웠으나, 우리의 항체를 적용했을 경우에는

아마도 이런 한계를 극복할 수 있을 것으로 기대한다. 결과적으로, 우리는 글리코실화-비의존적 결합을 할 수 있는 NT-proBNP 의 Gly63–Lys68 부분에 반응하는 항체를 개발하였고, 샌드위치 효소 면역측정법을 통해 NT-proBNP 의 글리코실화 여부와 관계없이 항체의 반응성이 있다는 것을 확인하였다. 마지막으로 우리는 이 항체와 펩타이드 미모톱을 이용한 경쟁적 효소 면역측정법을 개발하였다.

주요어: NT-proBNP, *O*-글리코실화, 파지 디스플레이, 펩타이드 미모톱, 효소 면역측정법

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