ABSTRACT

The humoral immunity has been evolved to protect the host. For an individual it is one of the greatest biological advantages to harbor effective repertoire of humoral immunity toward the various hostile agents like bacteria, virus or cancers. In this context, it has been a long-waiting unanswered question whether the centenarians do have a special repertoire of humoral immunity giving them the ability to survive longer than general population. Recently the phage display technology made it possible to define the circulating repertoire of humoral immunity. This study was designed to define the circulating repertoire of antibodies specific to centenarians.

To achieve this goal, the sera of three populations; centenarian, old (healthy volunteers between age 60 and 79), and young (healthy volunteers younger than 43), were collected. The IgG fractions were purified from the centenarians' sera, pooled and used to enrich specific phages from the phage display of combinatorial peptide library through biopanning. The phages reactive to the pooled IgG fractions of centenarians, but not to those of the other groups were selected by a phage enzyme immunoassay and their sequences were determined by nucleotide sequencing. Interestingly, phage clones encoding two homologous peptides, YSATLRY and YSPTLFY, were repeatedly found. In enzyme immunoassay, these two peptides, whether displayed on phage or chemically synthesized and conjugated to bovine serum albumin, reacted with centenarians' IgG fractions with much higher frequency than other groups' IgG fractions. As the antibody titers to these two peptides were highly correlated with each other, it was expected that

these two peptides actually represent the same epitope of an antigen.

To characterize and define the antigen represented by these two homologous peptides, polyclonal antibodies reactive to these peptides was purified from human sera. From the human cancer cell line LoVo, proteins were immunoprecipated by the purified polyclonal antibodies and subjected to SDS-polyacrylamide gel electrophoresis. The identity of the antigen was determined by mass spectrometry analysis and turned out to be the DNA-directed RNA polymerase II subunit RPB1 (RPB1)

Identity of the antigen was once more confirmed in immunoblot analysis using several antibodies specific to the antigen. For establishing the stable source of the antibody, a phage display of combinatorial antibody library was constructed using mRNA prepared from peripheral mononuclear cell fractions of volunteer possessing the antibody to the epitope. Through biopanning on YSATLRY, several clones reactive to both of the peptides, and the antibodies were successfully selected. Especially, one of these monoclonal antibodies had high affinity to the phosphorylated C-termial domain (CTD) of RPB1.

To extensively define the phosphorylated form of RPB1 CTD, we generated a synthetic antibody library by replacing the third heavy chain complementarity-determining region of an anti-HER2 antibody (trastuzumab) with artificial sequences of 7–18 amino acid residues. From this library, antibodies were selected that were specific to serine phosphopeptides representing typical phosphorylation patterns on the functional unit $(YSPTSPS)_2$ of the RPB1 CTD. Antibody clones pCTD-1stS2 and pCTD-2ndS2 showed specificity to peptides with phosphoserine at the second residues of the first or second heptamer repeat, respectively. Additional clones specifically reacted to peptides

with phosphoserine at the fifth serine of the first repeat (pCTD-1stS5), the seventh residue of the first repeat and fifth residue of the second repeat (pCTD-S7S5), or the seventh residue of either the first or second repeat (pCTD-S7). All of these antibody clones successfully reacted to RPB1 CTD in immunoblot analysis. Interestingly, in genome-wide chromatin immunoprecipitation sequencing analysis, pCTD-2ndS2 precipitated predominately RNA polymerase II on the exonic regions of genes, which suggests that the phosphoserine at the second residue of the second repeat on the functional unit $(YSPTSPS)_2$ is a mediator of exon definition.

In conclusion, we confirmed that centenarians do have a specific antibody in much higher frequency than the general population, and the antigen was identified as RPB1. We also have developed antibodies against its multiple phosphorylated form.

Keywords : Aging, centenarian, phage display, RNA polymerase II, RPB1, carboxy terminal domain (CTD), phosphorylation

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INTRODUCTION

1. Aging and Immune system

Life expectancy is growing longer and longer nowadays [1]. And, aging is associated with a decline in health, partially results in defects in immunity [2]. Conventional immunosenescence describes the decreased immune ability following aging. The complex process of immunosenescence affects both the innate and the adaptive aspects of human immune system [3]. In innate immune system, it is reported that the overall functions of NK, NKT, Mast, eosinophil are decreased [3]. It has been studied that not only the function of innate immunity but also, acquired immunity decreases during aging. As organism ages, the primary immune organ, thymus and bone marrow, decreases in its size and function, following the decrease of naïve B cell and T cell, resulting in weaker secondary immune response [4].

Especially, the humoral immunity has been evolved to protect the host. For an individual, it is one of the greatest biological advantages to harbor effective repertoire of humoral immunity toward the various hostile agents like bacteria, virus or cancers [5]. Observing the depression of age-related immune system in the standpoint of humoral immunity, diverse CD28+ positive T cells exist at newborn, but decreases by senescence proceeds. And aging leads homeostatic expension, resulting in decreased resistance to novel antigen. Moreover, reduced overall TCR diversity is observed during aging [6]. Thus, bone marrow, thymic tissue transplantation, or vaccination of hostile elements could be useful methodology for age-related immune therapy [7].

2. Centenarian

Centenarians represents the model of successful aging [8]. They not only maintain a longer lifespan than normal population, but also are resistant to senile disease such as cancer, dementia, diabetes and cardiovascular disease, and maintain both healthy physical and mental life. Thus, it is a great model system for discovering the basic senescence mechanism or longevity.

Recently, 'remodeling theory of aging', a novel concept, provides good explanation to these phenomena [9]. This theory started from the study of immune system of centenarians. It shows that some healthy elderly, including centenarians, harbors even increased immune function.

3. DNA-directed RNA polymerase II subunit RPB1 (RPB1) and its carboxy-terminal domain (CTD)

RNA polymerase II, which has molecular weight of 550kDa, is composed of 12 subunit proteins known as RPB1 to RPB12 [10]. Among them, RPB1 is the largest subunit and has carboxy-terminal domain (CTD). CTD has seven amino acid repeat of sequence $Y_1S_2P_3T_4S_5P_6S_7$ (Figure 1), this heptapeptide repeats 26 times in yeasts, 32 times in nematodes, 45 times in fruitflies, 52 times in mammals [11], [12], [13]. This repeats of the YSPTSPS motif can be phosphorylated simultaneously at multiple residues (except proline), which yields diverse serine-phosphorylation patterns [14]. It is expected that the post-translational modifications, including phosphorylation, isomerization, and

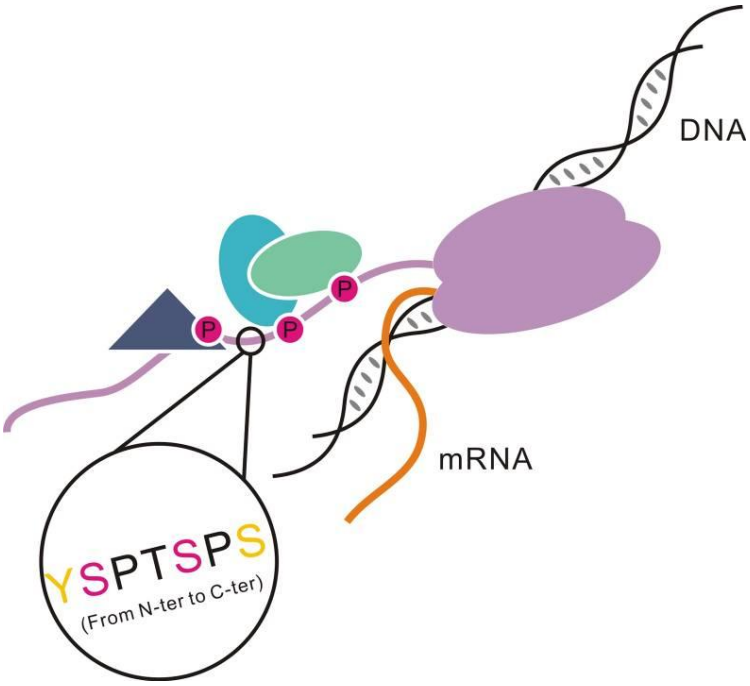
glycosylation occur frequently during transcription process, to regulate the function of RNA polymerase II.

Among them, phosphorylation is known to induce the extensive structural alterations of CTD. The repeated heptapeptides, more than 5 residue can be phosphorylated (Y_1, S_2, T_4, S_5, S_7) (figure 2). Especially, majority of the phosphorylation occurs on the serine residues [15], [16], on Ser2 and Ser5 are well characterized for their functional regulatory roles of CTD [17].

There exists number of studies of diverse phosphorylation forms of Ser2 and Ser5 on the CTD of RPB1 during transcription of protein coding genes (Figure 3). To take a closer look into this process, as transcription initiates, Ser5 is phosphorylated. Following the transcription progress through RNA coding region of the gene, Ser2 phosphorylation increases. As a result, Ser2 and Ser5 are both hyperphosphorylated. As transcription progresses through the poly (A) site of the gene, Ser5 phosphorylation gradually decreases. On the other hand, Ser2 phosphorylation continuously increases. While passing poly (A) site, Ser2 is dephosphorylated to terminate the transcription. After transcription is terminated, RPB1 CTD is not phosphorylated, so that it can re-initiate new cycle of transcription [18], [19].

Recently, the role of Ser7 phosphorylation has been studied extensively, that it participates in the transcription of multiple protein coding genes and snRNA [20]. Moreover, as high level of Ser7 phosphorylation is also found on the 3' end of T-cell receptor β (TCRβ) gene, it is expected that Ser7 phosphorylation also participates in the 3' end processing or transcription termination like Ser2 phosphorylation [21]. And it is studied that Ser7 phosphorylation participates in the transcription process and termination

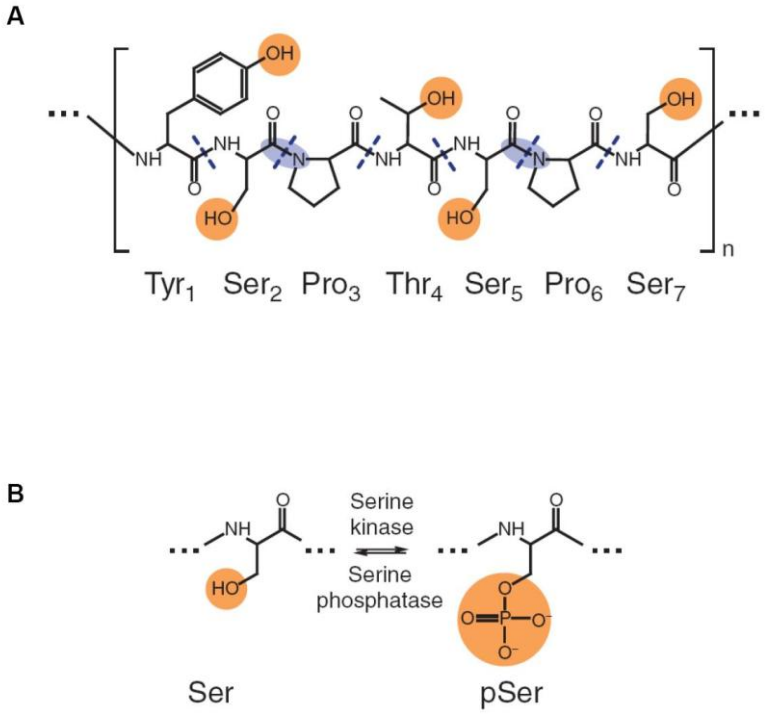
of human snRNA. Not to mention that this phenomena increases the overall complexity of CTD code, since multiple combinations of Ser2-Ser5-Ser7 or Ser2-Ser7 phosphorylation can take part in transcription elongation [13].



Mengmeng Z. et al. Nano Reviews. 2010;1: 5502

Figure 1. Model of the RNA polymerase II.

The RNA polymerase II is colored with purple. Different shapes bound to the CTD indicate various proteins that are recruited by the CTD. Magenta circles labeled with 'P' indicate phosphorylation on the CTD. One repeat in the black circle is zoomed in to show its primary sequence 'YSPTSPS'. The Ser2 and Ser5 (colored with magenta) are phosphorylated in each round of transcription, and Tyr1 and Ser7 (colored with yellow) are also detected as phosphorylation sites *in vivo*.



Wiley Interdiscip Rev RNA. 2013 Jan-Feb;4(1):1-16.

Figure 2. Structure representation of the RPB1 CTD heptad and its possible modifications.

A. structure formula of the canonical heptapeptide of the CTD. B. Representation of the serine phosphorylation. Small orange circles indicate hydroxyl group that can be phosphorylated or modified otherwise, big orange circle indicates phosphorylation, blue ellipses indicate the serine-prolone peptidyl-prolyl bonds that can have two isomeric conformations, blue dashed lines show visual separation between amino acid residues.

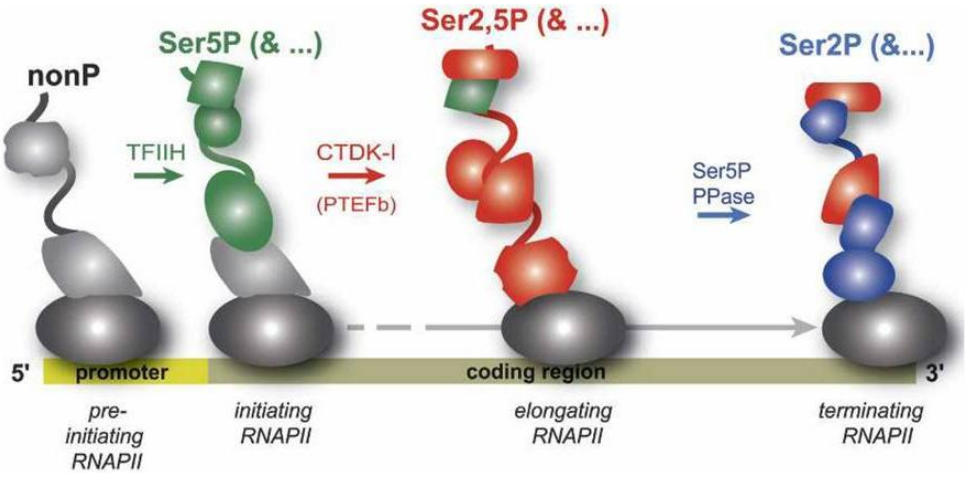


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Figure 3. RPB1 CTD phosphorylation patterns dictate which factors associate with RNA polymerase II.

RNA polymerase II (gray oval) is depicted at four positions along a gene, and at each position its CTD (wavy line) is a different color to indicate different phosphorylation states: Gray indicates nonphosphorylated repeats; green indicates Ser5P repeats; red indicates Ser2, 5P repeats (doubly phosphorylated); and blue indicates Ser2P repeats. Proteins bound to a type of repeat are indicated in the same color as the repeat.

4. Phage display technique

Phage display is a selection technique, which the foundation lies on the principle that library of peptide or protein variants is expressed on the outside of a phage virion, while the genetic material encoding each variant resides on the inside [22], [23], [24]. It allows rapid selection of a target molecule, based on binding affinity (antibodies, enzymes, cell-surface receptors, etc.) by an *in vitro* process called biopanning [25]. In 1985, George Smith initially demonstrated that antigenic polypeptides could be successfully displayed in a functional pIII viral coat protein fusion form on the surface of phage particles [26]. After that, several groups claimed that antibody fragments could also be displayed as viral coat proteins fusion forms [27], [28], [29] and opened the gate for the development of phage-displayed antibody libraries. Since then, all of the five phage coat proteins (pVIII, pIII, pVI, pVII and pIX) have been used for display [30], [31], [32], [33]. Among the five coat proteins, pIII and pVIII are most commonly used and well developed for the display of proteins or peptides, respectively [34]. The overall process of the antibody development using phage display is shown in figure 4.

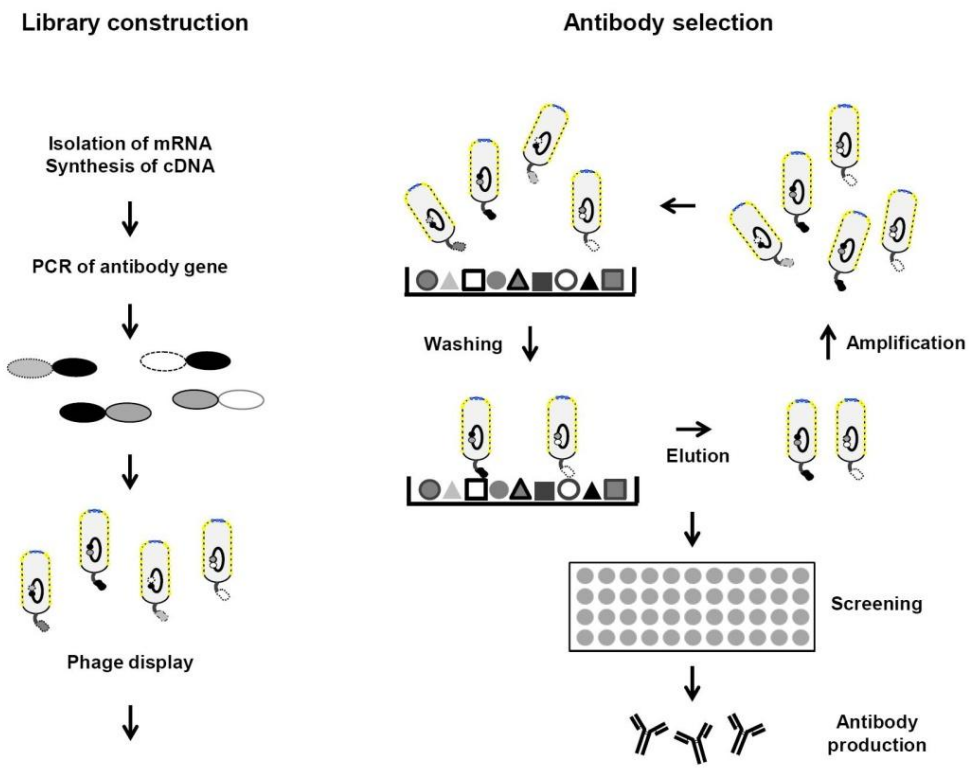

Figure 4. Overall process of the antibody development using phage display.

cDNA was reverse-transcribed from the total RNA. PCR was performed to amplify and assemble the antibody genes. Assembled antibody genes were cloned into phagemid vector. The vector is transformed into *E. coli*. With helper phage rescue, random combinatorial antibody library is displayed on the phage. This phage library contains diverse antibody fragments, which can be used to select positive antibody clones by biopanning. Biopanning is consisted with a series of binding, washing, elution, and amplification cycle. The resulting phage pool can be examined with enzyme immunoassay to confirm the reactivity of selected antibody clones.

4.1. Antibody phage library

Antibody library could roughly be categorized into 'immune library' and 'non-immune library'. Immune library is generated with antibody gene from immunized donor, while non-immune library uses naïve library generated with antibody gene from human or animal, or artificially generated synthetic library without immunization process [35].

Antibody genes can be obtained from antigen-immunized donor or synthesis *in vitro* [29]. Traditional antibody development often includes immunized library. Lymphocytes were collected from immune organs (bone marrow, spleen and etc.) and several rounds of PCR were done using set of various primers to obtain the antibody genes [36]. The coding sequence of the antibody, which is fused to the phage minor coat protein pIII, can be successfully displayed on the surface of phage.

In this study, we constructed antibody as single chain Fragment variable (scFv) region form. ScFv is an antibody, which consisted with heavy (V_H) and light (V_L) chain variable region. Rearranging heavy chain V_H and light chain V_L gene segments *in vitro* and introducing varying sequences of artificial complementary determining regions (CDRs) via overlap PCR using randomizing primers are the major parts of the antibody library construction. Generated fragment of the antibody is cloned into phagemid vector to transform into *E.coli*. Antibodies and most eukaryotic proteins require disulfide bond formation for stability. *E.coli* periplasm provides oxidizing environment, which is an appropriate place for correct antibody folding.

Antibody displaying phages are produced by transfection with VCSM13 helper phage for precise replication and assembly of phage particles [37]. Antibodies with diverse CDR combinations would be produced, since final library size typically reaches 10^9. As it takes

only a few weeks to engineer target-specific antibody, phage display is an efficient technique for antibody development.

4.2. Synthetic antibody library

Immune library has great advantage, as it grants a promising probability of a higher affinity antibody discovery, by boosting antibody generation with antigen immunization process. However, it is relatively complicated and time-consuming process, as it starts with immunization, purifying RNA from retrieved organ, cDNA synthesis, followed by multiple recombinant PCR progress. On the other hand, synthetic library, which could be synthesized by artificial recombination of antibody gene, has capability of simplifying the complex nature of immune library and also, the size of the library could be manipulated regarding the purpose of the experiment. In addition, it has high stability and expression rate of the antibody as well as providing simplicity in optimization and engineering [35], [38]. Recently, human antibody against germline repertoire being sequenced, grants active production of germline-derived CDR sequence recombined synthetic library [35]. Germline repertoire can be classified by sequence homology consist with 6 subfamily grouped about 50 V_H (Heavy chain variable region) germline gene [39], [40], seven subfamily grouped about 40 functional V_L (Light chain variable region) kappa gene [41], [42], 10 subfamily grouped about 30 functional V_L lambda gene [43], [44], [45]. Various combinations of them enabled the active research in the generation of numerous tailored synthetic libraries.

The use of synthetic antibody libraries is especially valuable for developing antibodies to phosphorylated protein motifs because it is difficult to produce these antibodies from or

naïve or immunized sources [46]. Previously, one anti-phosphopeptide motif antibody was successfully engineered into multiple antibodies reactive to diverse phosphopeptides by inserting an anion-binding nest in HCDR2 and by randomizing HCDR3 residues [47].

In this study, we constructed a synthetic scFv library with a trastuzumab backbone [48], [49] and artificial HCDR3 with 7-18 amino acid residues (Figure 16). We adopted the anti-HER2 antibody trastuzumab as the scaffold because it can harbor reactivity to multiple antigens by modifying its CDRs. Previously, trastuzumab has been engineered to display substantial affinity to VEGF while maintaining its reactivity to HER-2 by introducing mutations on its LCDRs [50].

4.3. Combinatorial peptide library

Random peptide library, which is introduced around same time with the phage display technique, is capable of generating diverse peptide libraries by inserting randomly synthesized peptide into the coat protein of phage. And by phage display technique, antigen determining region, which interacts with certain antibody, could be identified [25]. Also, the phage display of combinatorial peptide library made it possible to enrich peptides reactive to antibodies inside human body [51].

Random peptide libraries consist of linear heptapeptide (7mer) and dodecapeptide (12mer) libraries, as well as a disulfide-constrained heptapeptide (C7C mer) library. The randomized segment of the C7C library is flanked by a pair of cysteine residues, which are oxidized during phage assembly to a disulfide linkage, resulting in the displayed peptides being presented to the target as loops. All of the libraries have complexities in excess of 2 billion independent clones (Table 1).

Table 1. Types of combinatorial peptide library.

Peptide library	Sequence	Library complexity (expected complexity, achieved complexity)
Random peptide 12mer X_{12}GGGS	NNK NNK NNK NNK NNK NNK NNK NNK NNK NNK NNK NNK X X X X X X X X X X X X	4.1 X 10^{15}, 1.9 X 10^{9} independent clones
Random peptide 7mer X_7GGGS	NNK NNK NNK NNK NNK NNK NNK X X X X X X X	1.28 X 10^{9}, 2.0 X 10^{9} independent clones
C7C mer ACX$_7$CGGGS SS	GCT TGT NNK NNK NNK NNK NNK NNK NNK TGC ALA CYS X X X X X X X CYS	1.28 X 10^{9}, 3.7 X 10^{9} independent clones

4.4. Biopanning

Biopanning is enrichment and selection process of specific binding partners from phage display library. During this process, antibody or peptide displaying phage, which recognize and specifically binds to target protein, is amplified by repetitive panning-selection. In biopanning process, target proteins are attached to various solid supports, like magnetic beads [52], column matrix [53], nitrocellulose [54] or to a larger scale, plastic surfaces in the form of polystyrene tubes [55], or 96 well polystyrene microtiter plates [56].

Generally, phage library, each displaying a different antibody or peptide, is exposed to the plate or to the bead, coated with the target molecule. Throughout the washing step, unbound phages are washed away. Repetitive rounds of panning are performed with increasing the number of washing steps. Specifically-bound phage clones are eluted by lowering the pH, and amplified. After 3-4 rounds, individual clones are isolated and sequenced.

The size of library (the number of clones) could be major parameter to selecting good binder. Nowadays, phage library is capable of generating the complexity over the size of 10^{11}. However, due to the maximum transformation efficiency of *E.coli* is currently about $10^{9\text{-}10}$cfu/ml, it is not possible to increase the library size unlimitedly [57].

In this reason, library design and construction have great effects on the binder selection process. There are various selection methods are demonstrated. For better selecting binders and removing non-binders, matrix types, blocking agents, elution and infection methods, can be modified [58]. Also, high-throughput screening of antibody sequences using next-generation sequencing (NGS) would be required to select antibody clones [59].

Converging biopanning and NGS are emerging as the next generation technique for screening clones [60].

MATERIALS AND METHODS

Cell culture

HEK 293T and LoVo cells were obtained from the American Type Cell Collection (Manassas, VA, USA) and grown in Dulbecco's Modified Eagle Medium (Welgene, Seoul, South Korea) supplemented with 10% fetal bovine serum (GIBCO), 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C in a humidified atmosphere of 5% CO_2.

HEK 293F cells (Invitrogen, Carlsbad, CA, USA) were grown in FreeStyle™ 293 Expression medium (GIBCO, Grand Island, NY, USA), containing 100 U/mL penicillin and 100 μg/mL streptomycin at 37 °C in air containing 7% CO_2 and 95% relative humidity (RH) on an orbital shaking incubator (Minitron, INFORS HT, Bottmingen, Switzerland) at 135 rpm.

Peptide synthesis and conjugation

All peptides used in this study were synthesized by Peptron, Inc. (Daejeon, South Korea). The peptides were synthesized and conjugated to BSA as described previously [61].

Preparation of human serum and antibodies

Sera were collected from 45 centenarians [62] and 50 healthy volunteers [63] (Table 2). The study was approved by the Institutional Review Board (IRB) of Samsung Medical Center (200412005). Briefly, 11, 22, and 12 centenarians were recruited from rural areas of Kyungsang, Chunra, and Cheju provinces in South Korea, respectively. A medical survey team visited the houses of centenarians and collected blood samples as a part of

the survey procedure. The informed consent form was signed either by the subjects themselves or close relatives. To prepare polyclonal antibodies (pAbs) to YSATLRY, sera were collected from 59 additional healthy volunteers. The study was approved by the IRB of Seoul National University Hospital (C-0710-006-221). Written informed consent was obtained from all volunteers in accordance with the Declaration of Helsinki. Personal identifiers were removed, and data were analyzed anonymously.

Preparation of electrocompetent cell

A single colony of *E. coli* ER2738 (New England Biolabs, Beverly, MA, USA) was inoculated to 15 ml of prewarmed SB medium containing 12 μg/ml of tetracycline and incubated overnight at 37 ℃. The next day, 2.5 ml of the culture was transferred into 500 ml SB medium containing 10 ml of 20 % (w/v) glucose and 5 ml of 1 M $MgCl_2$ and shook at 250 rpm and 37 ℃ until the optical density (OD) at 600 nm reaches 0.8~1.0. The culture was poured into prechilled centrifuge bottle and incubated 15 min on ice. The culture was separated into supernatant and pellet by centrifugation at 3,000 g for 20 min at 4 ℃. The supernatant was discarded and the pellet was resuspended in 300 ml of prechilled 10 % (w/v) glycerol solution. The resuspension was spun as before. After three times of pellet washing, the supernatant was discarded and the pellet was resuspended in the small volume of glycerol solution remnant, and stored at -80 ℃.

Preparation of helper phage

A single plaque of VCSM13 (Stratagene, La Jolla, CA, USA) was infected to the *E. coli* ER2738 culture which was incubated in 10 ml of SB medium and reached $OD_{600} = 1.0$.

Infected culture was incubated for 2 hr and transferred to 500 ml of prewarmed SB medium containing kanamycin to a final concentration of 70 μg/ml and cultured overnight at 37 ℃. After the culture was centrifuged at 3,500 rpm for 15 min, the supernatant was incubated at 70 ℃ for 20 min and spun at 3,500 rpm for 15 min again. The supernatant was isolated and stored at 4 ℃.

Biopanning for discovering the peptides bind to centenarians' IgG fractions.

The phage display of combinatorial peptide libraries were used as random peptide 12mer, random peptide 7mer and C7Cmer from Ph.D. Phage Display Peptide Library (New England Biolabs, Ipswich, MA, USA).

To enrich specific binding peptides from these random peptide libraries (Ph.D. Phage Display Peptide Library, New England Biolabs), a total of three rounds of biopanning were performed as described previously [37].

IgG fractions from centenarians were immobilized to protein G agarose bead (Santacruz) at room temperature for 2 hr. IgG coated beads were washed with phosphate-buffered saline (PBS) and blocked with 3% BSA in PBS (w/v) for 1 hr at room temperature on a rotator. After blocking, beads were incubated with phage-displayed peptides for 2 hr at room temperature on a rotator and then washed with 0.05% Tween-20 in PBS (v/v, PBST) to remove unbound phage and the number of washes was increased to three times in subsequent rounds. After washing, 0.1 M glycine-HCl (pH 2.2) was added to the beads and incubated for 10 min at room temperature for the elution of bound phages from the beads. Eluate was neutralized by adding 2 M Tris-Cl (pH 9.1). Afterwards, the eluted phages were used to infect *E. coli* strain ER2738 (New England Biolabs) culture, and the

peptide-displaying phages were rescued by adding helper phages (VCSM13, Stratagene, La Jolla, CA, USA). Individual phage clones were selected from the output titration plate from the last round of biopanning, and peptide-displaying phages were prepared for the phage enzyme immunoassay, as described previously [37].

To select binders, the reactivity of peptide-displaying phage was tested in phage enzyme immunoassay. The phage clones with positive signals were selected ($O.D_{405} \geq 0.5$) and their nucleotide sequences were determined.

Individual phage enzyme immunoassay with selected phage clones.

Enzyme immunoassay using phages displaying peptides was performed against human IgG to analyze the selected clones from biopanning. Microtiter 96-well plates (Corning Costar Corp., Cambridge, MA, USA) were coated with anti-human IgG Fab specific (Sigma, St. Louis, MO, USA) per well for overnight at 4 ℃ and blocked with 3% BSA in PBS. Each individual human serum diluted in 3% BSA in PBS was incubated for 2 hr at 37 ℃. After washing with 0.05% PBST three times, each phage cultures were mixed with an equal volume of 6% BSA in PBS and incubated for 2 hr at 37 ℃. After washing with 0.05% PBST three times, plates were incubated with HRP conjugated anti-M13 antibody (GE Healthcare Life Sciences, Piscataway, NJ, USA). Washing steps were repeated five times. 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS, Amresco, Solon, OH, USA) in 0.05M Citric acid buffer (pH 4.0) and 1.0 % H_2O_2 were added to each well. Optical density (OD) was measured at 405 nm with microplate photometer (Labsystems Multiskan, Thermo Fisher Scientific, Rockford, IL, USA).

Preparation of the polyclonal antibodies (pAbs) from human serum.

To find the candidate showing high affinity to YSATLRY and YSPTLFY, peptides were synthesized with the addition of linker sequence (C'-Gly-Gly-Gly-Ser-Cys) and conjugated to BSA. A YSATLRYGGGSC-cross-linked affinity column was prepared using a Sulfolink kit (Pierce Biotechnology, Inc., Rockford, IL, USA), and pAbs were purified from five volunteers' sera (Volunteer #7, #11, #19, #47, and #50) according to the manufacturer's instructions.

Competetion enzyme immunoassay

Microtiter 96-well plates (Corning Costar Corp., Cambridge, MA) were coated with YSATLRYGGGSC-BSA conjugates each well for overnight at 4 ℃ and blocked with 3% BSA in PBS. A mixture of pAbs and peptides was added to each well and the plates were incubated for 2 hr at 37 ℃. After washing with 0.05% Tween-20 in PBS (v/v, PBST) three times, plates were incubated with horseradish peroxidase (HRP) conjugated anti-human IgG antibody (GE Healthcare Life Sciences, Piscataway, NJ, USA). Washing steps were repeated three times. 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS, Amresco, Solon, OH, USA) in 0.05M Citric acid buffer (pH 4.0) and 1.0 % H_2O_2 were added to each well. Optical density (OD) was measured at 405 nm with microplate photometer (Labsystems Multiskan, Thermo Fisher Scientific, Rockford, IL, USA).

YSATLRYGGGSC and irrelevant control peptide (LPCYTDHICYSSGGGSC) were used for compete with pAbs. All the peptides used in this study were synthesized from Peptron,Inc. (Daejeon, South Korea)

Mass spectrometry analysis.

Mass spectrometry was performed by the Peptide Library Support Facility, POSTECH (Pohang, South Korea) on request. Samples were analyzed using the Applied Biosystems 4700 proteomics analyzer (SCIEX, Forster City, CA, USA). Protein identification using peptide sequences from LC–MS/MS samples was performed using MASCOT software as described previously [64].

Human naïve antibody library construction and biopanning.

A phage display of combinatorial antibody library of single chain variable fragment variable forms (scFvs) was constructed by acquiring total RNA isolated from peripheral mononuclear cell fractions of volunteer #19. Peripheral mononuclear cell fractions were isolated from volunteer #19's blood using Ficoll-Paque PLUS (GE healthcare Life Sciences, Piscataway, NJ, USA) following the manufacturer's instructions.

ScFv antibody library was generated with long linker (GGSSRSSSSGGGGSGGGG) using phage display technique, as described previously [37] (Figure 5 and Table 3).

To enrich specific binders from the library, a total of four rounds of biopanning were performed as described previously [37]. Three micro gram of YSATLRYGGGSC-BSA conjugates was conjugated to 5.0 x 10^6 paramagnetic beads (DynaBeads, Invitrogen) following the manufacturer's instruction. Rest of the procedures are performed as described in 'Biopanning for discovering the peptides bind to centenarians' IgG fractions'.

Enzyme immunoassay.

Microtiter 96-well plates (Corning Costar Corp., Cambridge, MA) were coated with

antigen for overnight at 4 ℃ and blocked with 3% BSA in PBS. Primary antibody was diluted with 3% BSA in PBS and incubated for 2 hr at 37 ℃. After washing with 0.05% Tween-20 in PBS (v/v, PBST) three times, plates were incubated with HRP conjugated secondary antibody. Washing steps were repeated three times. 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS, Amresco, Solon, OH, USA) in 0.05M Citric acid buffer (pH 4.0) and 1.0 % H_2O_2 were added to each well. Optical density (OD) was measured at 405 nm with microplate photometer (Labsystems Multiskan, Thermo Fisher Scientific, Rockford, IL, USA).

Immunoprecipitation.

Cells were lysed with lysis buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% (v/v) Triton X-100, 0.25% (v/v) Na-deoxycholate, 1mM EDTA, 1× protease inhibitor cocktails (Amresco, Solon, OH, USA), 1× phosphatase inhibitor cocktails (Roche, Basel, Switzerland)), and incubated with anti-YSATLRY polyclonal antibodies or scFv-Fc fusion proteins, which were purified from human sera in the presence of protein A agarose beads (Repligen, Waltham, MA, USA). After incubation, the beads were washed four times with lysis buffer and immunoprecipitates were boiled with SDS sample buffer, loaded onto SDS-PAGE gels.

Immunoblot.

Immunoprecipitated protein or whole cell lysates were subjected to electrophoresis on a NuPage 4-12 % Bis-Tris gel (Invitrogen, Carlsbad, CA, USA) and resolved proteins were transferred to nitrocellulose membrane. Transferred membrane was blocked with 5% non-

fat dry milk (BD Biosciences, Sparks, MD, USA) in 0.1% Tween-20 in TBS (v/v, TBST) at room temperature for one hr. The membrane was incubated with primary antibodies (anti-pan CTD, phosphorylated serine 2 and serine 5 were from Abcam plc., Cambridge Science Park, Cambridge, UK) diluted in 5% non-fat dry milk-0.1% TBST for overnight at 4 ℃. After washing four times with 0.1% TBST, membrane was incubated with HRP conjugated secondary antibodies (anti-mouse IgG Fc specific-HRP was from Sigma, Spruce St. St. Louis, MO, anti-rabbit IgG Fc-HRP and anti-rat IgG H+L specific-HRP were from Jackson ImmunoResearch Inc., West Grove, PA, USA) diluted in 5% non-fat dry milk-0.1% TBST for one hr at room temperature. The membrane was washed four times with 0.1% TBST, and protein was visualized by SuperSignal Pico West chemiluminescent substrate (Thermo Fisher Scientific, Rockford, IL, USA).

Determination of antinuclear antibody (ANA) IgG level in the sera

The level of ANA IgG in the sera was determined using a commercially available enzyme immunoassay kit (Abnova, Taipei, Taiwan) following the manufacturer's instructions. Briefly, sera were diluted 1:21 with the sample diluent included in the kit. Then, 100 μl each of the diluted sera, calibrator, and controls were added into separate wells and incubated at room temperature for 20 min. After washing, enzyme conjugate was added into each well. The plate was then incubated at room temperature for 20 min before washing. TMB substrate solution was added into each well and the plate was incubated at room temperature for 10 min. After the stop solution was added, the OD was measured at 450 nm with a microplate spectrophotometer (Labsystems Multiskan, Thermo Fisher Scientific). OD values were converted to antibody indices according to the

manufacturer's instructions. When the antibody index was higher than 1.1, the sample was considered to have a detectable level of ANA IgG.

Construction of the synthetic scFv library

Amino acid sequences of the trastuzumab variable regions (V_H and V_L) were obtained from the PDB database (1N8Z). The sequence encoding the V_L domain, a linker (Glycine–Glycine–Glycine–Glycine–Serine)$_3$, and the part of the V_H domain from FR1 to FR3 was chemically synthesized (Integrated Device Technology, Inc., San Jose, CA, USA). The region encoding partial FR3, synthetic HCDR3, and FR4 of V_H was generated by PCR using degenerate primers and Her-VH-R primer (5'-CCGGCCGGCCTGGCCGGAGGACACGGTCACCAGG-3'). Ten degenerate primers were used to introduce artificial sequences into HCDR3 (Table 4). Two DNA fragments were combined by overlap extension PCR using HAX-VL-F (5'-GGCCCAGGCGGCCGACATCC-3') and Her-VH-R primers to generate the scFv gene with *Sfi*I restriction sites at both termini. The genes encoding scFv and pComb3XSS phagemid vector were subjected to *Sfi*I restriction enzyme digestion and subsequent ligation. The recombinant vector was then transformed into *E. coli* strain ER2738 (New England BioLabs, Ipswich, MA, USA), as described previously [37]. After overnight culture, a phage-displayed combinatorial antibody library was prepared [37].

Biopanning and phage enzyme immunoassays

To enrich specific binders from the library, five rounds of biopanning were performed as described previously [37]. One hundred and fifty micrograms of each phospho-

YSPTSPSYSPTSPS-BSA conjugate was mixed with 3.0×10^8 paramagnetic beads (M-270 Expoxy DynaBeads, Invitrogen) following the manufacturer's instructions. After overnight incubation at room temperature on a rotator, the beads were washed four times with 0.5% BSA in phosphate-buffered saline (PBS) and blocked with 3% BSA in PBS (w/v). The phage-displayed combinatorial antibody library was added to the beads and incubated for 2 h at room temperature on a rotator. The beads were washed once with 0.05% Tween-20 in PBS (PBST, v/v) for the first round, increasing to three washes in subsequent rounds. After washing, 0.1M glycine-HCl (pH 2.2) was added to the beads, and the mixture was incubated for 10 min at room temperature to elute bound phage from the beads. The eluate was neutralized by adding 2M Tris-Cl (pH 9.1), and the eluted phages were transduced into *E. coli* strain ER2738. Phage displaying scFv were rescued by adding VCSM13 helper phages (Stratagene, La Jolla, CA, USA). Individual phage clones were selected from the output titration plate from the last round of biopanning, and scFv-displaying phages were prepared for the phage enzyme immunoassay as described previously [37].

To select binders, the reactivity of antibody-displaying phage was tested in phage enzyme immunoassays using phage-containing culture supernatant and horseradish peroxidase (HRP) conjugated anti-M13 antibody (GE Healthcare, Piscataway, NJ, USA). The phage clones with positive signals were selected ($O.D_{405} \geq 0.5$), and their nucleotide sequences were determined as described previously [37].

Expression and purification of scFv-Fc fusion proteins

An expression vector modified from pCEP4 (Invitrogen) was used for cloning and has

been described previously [65], [66]. The vector carries an expression cassette composed of the leader sequence of the human Ig κ-chain, two *Sfi*I sites for insertion of the antibody gene of interest, the hinge region of human IgG_1, and the C_{H2}–C_{H3} domains of rabbit IgG. The phagemid DNA of selected clones and the expression vector were digested with *Sfi*I, and the scFv genes were cloned into the expression vector. Recombinant pCEP4 was transfected into HEK 293F cells using 25-kDa linear polyethylenimine (Polysciences, Warrington, PA, USA) as described previously [67]. Overexpressed scFv-Fc fusion proteins were purified by affinity chromatography using protein A sdpharose columns (Repligen, Waltham, MA, USA) according to the manufacturer's instructions.

Chromatin Immunoprecipitation assay

The ChIP assay was performed based on the method of Lee et al. with slight modifications [68]. Briefly, HeLa cells were cross-linked in a solution of 1% formaldehyde in PBS for 5 min at room temperature. The cross-linking reaction was stopped by adding glycine to a final concentration of 0.125 M. The cells were harvested and washed twice with cold PBS, and cytosolic fractions were eliminated with buffer A (5 mM PIPES [pH 8.0], 85 mM KCl, 0.5% NP-40, protease inhibitor cocktail [GenDEPOT, Katy, TX, USA]). Nuclear pellets were resuspended in buffer B (100 mM Tris-Cl [pH 8.1], 1% SDS, 10 mM EDTA, protease inhibitor cocktail), and the chromatin was sheared with an S220 focused-ultrasonicator (Covaris, Woburn, MA, USA). The prepared chromatin fraction (500 μg total sheared DNA/sample) was diluted 1/10 in IP buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-Cl [pH 8.1], 167 mM NaCl, and protease inhibitor cocktail) and incubated with 200 μg of scFv-Fc fusion proteins (pan or

phosphorylation-specific RNA polymerase II CTD antibodies) overnight at 4℃. Samples were incubated for 2–4 hours at 4℃ with protein A or G beads pre-coated with salmon sperm DNA. Then the beads were washed with TSE150 (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-Cl [pH 8.1], 150 mM NaCl), TSE500 (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-Cl [pH 8.1], 500 mM NaCl), Buffer III (0.25 M LiCl, 1% NP-40, 1% sodium deoxycholate, 1 mM EDTA, 10 mM Tris-Cl [pH 8.1]), and two times with TE (pH 8.0), for 10 min in each solution. Bead-bound chromatin was eluted with elution buffer (1% SDS, 0.1 M $NaHCO_3$ [pH 8.0]) and incubated overnight at 65℃ with 200mM NaCl for reversal of cross-linking. Five hundred microliters of sample were incubated at 50℃ after addition of 10 μl of 0.5 M EDTA, 20 μl of 1 M Tris (pH 6.5), and 4 μl of Proteinase K (20 mg/ml), and purified with phenol/chloroform/isoamyl alcohol, as described previously [69]. Nucleic acids were precipitated by centrifugation for 30 min at 4℃ after mixing the sample with 1 μl glycogen solution (20 mg/ml), 20 μl 5M NaCl, and 500 μl isopropanol. Purified nucleic acid pellets were washed with 70% ethanol, dried and dissolved in nuclease-free water.

ChIP sequencing

Sequencing and analysis of ChIPed DNA fragments were performed by Macrogen (Seoul, South Korea). Briefly, DNA fragments were ligated to a pair of adaptors for sequencing on Hiseq® 2500 sequencing system (Illumina, San Diego, CA, USA). The ligation products were size-fractionated on a 2% agarose gel to obtain 200–300-bp fragments and PCR-amplified for 18 cycles. Each library was diluted to 8 pM for 76 cycles of single-read sequencing on the Hiseq® 2500 following the manufacturer's recommended protocol.

After read trimming, Bowtie V1.1.1 (read mapping), Picard V1.133 (remove duplicates), MACS 2 V2.1.0.20150420 (peak calling), and ChIPseeker (peak annotation) were used for downstream analysis.

Table 2. Age and gender distribution of subjects

Age group	Ages included		Gender		Age	
			N	%	Mean±SD	Range
Centenarians[62]	≥100	M	6	13.3	101.83±1.33	101-104
		F	39	86.7	101.74±1.46	100-105
		Total	45	100.0	101.76±1.43	100-105
Old[63]	60~79	M	11	44	65.55±4.97	61-79
		F	14	56	65.64±4.36	60-72
		Total	25	100.0	65.60±4.54	60-79
Young[63]	≤43	M	7	28	34.00±5.20	27-41
		F	18	72	34.67±3.88	29-43
		Total	25	100.0	34.48±4.18	27-43

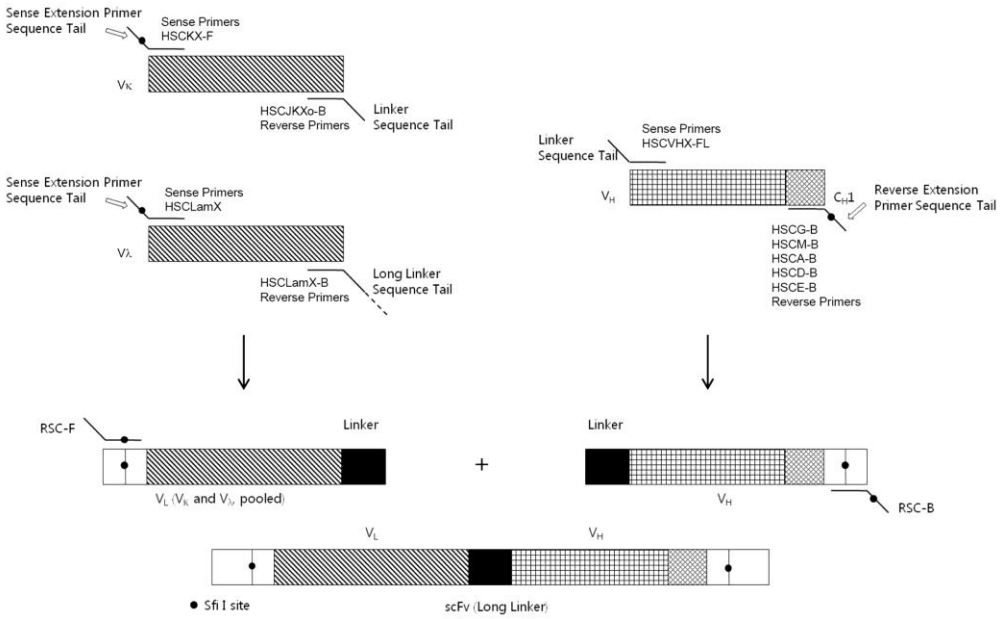

Figure 5. Construction of human scFv libraries (long linker).

Human V_κ, V_λ and V_H sequences were amplified 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. Each sense primer has a 5' sequence tail that contains a *Sfi*I site and is recognized by the sense extension primer used in the second-round PCR. Each reverse primer has a linker sequence tail that is used in the overlap extension. Human V_L and V_H fragments were combined in the second-round PCR. The sense and reverse extension primers recognize the sequence tails that were generated in the first round of PCR.

Table 3. Primers for Vκ, V_λ and V_H of human scFv libraries with long linker.

V_H Primers, 5' Sense, Long Linker		
HSCVH1-FL	5′	GGT GGT TCC TCT AGA TCT TCC TCC TCT GGT GGC GGT GGC TCG GGC GGT GGT GGG CAG GTG CAG CTG GTG CAG TCT GG 3′
HSCVH2-FL	5′	GGT GGT TCC TCT AGA TCT TCC TCC TCT GGT GGC GGT GGC TCG GGC GGT GGT GGG CAG ATC ACC TTG AAG GAG TCT GG 3′
HSCVH35-FL	5′	GGT GGT TCC TCT AGA TCT TCC TCC TCT GGT GGC GGT GGC TCG GGC GGT GGT GGG GAG GTG CAG CTG GTG SAG TCT GG 3′
HSCVH3a-FL	5′	GGT GGT TCC TCT AGA TCT TCC TCC TCT GGT GGC GGT GGC TCG GGC GGT GGT GGG GAG GTG CAG CTG KTG GAG TCT G 3′
HSCVH4-FL	5′	GGT GGT TCC TCT AGA TCT TCC TCC TCT GGT GGC GGT GGC TCG GGC GGT GGT GGG CAG GTG CAG CTG CAG GAG TCG GG 3′
HSCVH4a-FL	5′	GGT GGT TCC TCT AGA TCT TCC TCC TCT GGT GGC GGT GGC TCG GGC GGT GGT GGG CAG GTG CAG CTA CAG CAG TGG GG 3′
V_H Primers, 3' Reverse, Short and Long Linker		
HSCG1234-B	5′	CCT GGC CGG CCT GGC CAC TAG TGA CCG ATG GGC CCT TGG TGG ARG C 3′
HSCM-B	5′	CCT GGC CGG CCT GGC CAC TAG TAA GGG TTG GGG CGG ATG CAC TCC C 3′
HSCA-B	5′	CCT GGC CGG CCT GGC CAC TAG TGA CCT TGG GGC TGG TCG GGG ATG C 3′
HSCD-B	5′	CCT GGC CGG CCT GGC CAC TAG TCA CAT CCG GAG CCT TGG TGG GTG C 3′
HSCE-B	5′	CCT GGC CGG CCT GGC CAC TAG TGA CGG ATG GGC TCT GTG TGG AGG C 3′
Vκ Primers, 5' Sense, Short and Long Linker		
HSCK1-F	5′	GGG CCC AGG CGG CCG AGC TCC AGA TGA CCC AGT CTC C 3′
HSCK24-F	5′	GGG CCC AGG CGG CCG AGC TCG TGA TGA CYC AGT CTC C 3′
HSCK3-F	5′	GGG CCC AGG CGG CCG AGC TCG TGW TGA CRC AGT CTC C 3′
HSCK5-F	5′	GGG CCC AGG CGG CCG AGC TCA CAC TCA CGC AGT CTC C 3′
Vκ Primers, 3' Reverse, Short and Long Linker		
HSCJK14o-B	5′	GGA AGA TCT AGA GGA ACC ACC TTT GAT YTC CAC CTT GGT CCC 3′
HSCJK2o-B	5′	GGA AGA TCT AGA GGA ACC ACC TTT GAT CTC CAG CTT GGT CCC 3′
HSCJK3o-B	5′	GGA AGA TCT AGA GGA ACC ACC TTT GAT ATC CAC TTT GGT CCC 3′
HSCJK5o-B	5′	GGA AGA TCT AGA GGA ACC ACC TTT AAT CTC CAG TCG TGT CCC 3′
Vλ Primers, 5' Sense, Short and Long Linker		
HSCLam1a	5′	GGG CCC AGG CGG CCG AGC TCG TGB TGA CGC AGC CGC CCT C 3′
HSCLam1b	5′	GGG CCC AGG CGG CCG AGC TCG TGC TGA CTC AGC CAC CCT C 3′
HSCLam2	5′	GGG CCC AGG CGG CCG AGC TCG CCC TGA CTC AGC CTC CCT CCG T 3′
HSCLam3	5′	GGG CCC AGG CGG CCG AGC TCG AGC TGA CTC AGC CAC CCT CAG TGT C 3′
HSCLam4	5′	GGG CCC AGG CGG CCG AGC TCG TGC TGA CTC AAT CGC CCT C 3′
HSCLam6	5′	GGG CCC AGG CGG CCG AGC TCA TGC TGA CTC AGC CCC ACT C 3′
HSCLam78	5′	GGG CCC AGG CGG CCG AGC TCG TGG TGA CYC AGG AGC CMT C 3′
HSCLam9	5′	GGG CCC AGG CGG CCG AGC TCG TGC TGA CTC AGC CAC CTT C 3′
HSCLam10	5′	GGG CCC AGG CGG CCG AGC TCG GGC AGA CTC AGC AGC TCT C 3′
Vλ Primers, 3' Reverse, Short and Long Linker		
HSCJLam1236	5′	GGA AGA TCT AGA GGA ACC ACC GCC TAG GAC GGT CAS CTT GGT SCC 3′
HSCJLam4	5′	GGA AGA TCT AGA GGA ACC ACC GCC TAA AAT GAT CAG CTG GGT TCC 3′
HSCJLam57	5′	GGA AGA TCT AGA GGA ACC ACC GCC GAG GAC GGT CAG CTS GGT SCC 3′
Overlap Extension Primers		
RSC-F(sense)	5′	GAG GAG GAG GAG GAG GAG GCG GGG CCC AGG CGG CCG AGC TC 3′
RSC-B(reverse)	5′	GAG GAG GAG GAG GAG GAG CCT GGC CGG CCT GGC CAC TAG TG 3′

Table 4. Ten degenerate primers used to amplify HCDR3 derivatives

Primer ID	Sequence
H7-F	5′-GCC GTG TAC TAC TGC TCC AGA NNK NNK NNK NNK TTC GAC TAC TGG GGC CAG GGC ACA CTA-3′
H9-1-F	5′-GCC GTG TAC TAC TGC TCC AGA NNK NNK NNK NNK TAC GBT ATG GAC GTG TGG GGC CAG GGC ACA CTA-3′
H9-1-F	5′-GCC GTG TAC TAC TGC TCC AGA NNK NNK NNK NNK NNK NNK TTC GAC TAC TGG GGC CAG GGC ACA CTA-3′
H10-1-F	5′-GCC GTG TAC TAC TGC TCC AGA NNK NNK NNK NNK NNK TAC GBT ATG GAC GTG TGG GGC CAG GGC ACA CTA-3′
H10-2-F	5′-GCC GTG TAC TAC TGC TCC AGA XXXXXXX TTC GAC TAC TGG GGC CAG GGC ACA CTA-3′
H12-F	5′-GTG TAC TAC TGC TCC CGC GRT SBT XXXXXXX TTC GAC TAC TGG GGC CAG GGC ACC CTG-3′
H14-F	5′-GCC GTG TAC TAC TGC TCC AGA GRT SBT XXXXXXX DAT GST ATG GAC GTG TGG GGC CAG GGC ACA CTA-3′
H16-F	5′-GCC GTG TAC TAC TGC TCC AGA GNT VBT VKT XXXXXXX KMT DAT GST ATG GAC GTG TGG GGC CAG GGC ACA CTA-3′
H18-F	5′-GCC GTG TAC TAC TGC TCC AGA BNT VBT VKT XXXXXXX TMT TMT KMT DAT GST ATG GAC GTG TGG GGC CAG GGC ACA CTA-3′
H18C-F	5′-GTG TAC TAC TGC TCC CGC GNT VBT VKT X TGC XXXX TGC TMT TMT KMT DAT GST ATG GAC GTG TGG GGC CAG GGC ACC CTG-3′

N-A, C, G, T; V-A, G, C; B-C, G, T; K-G, T; M-A, C; D-A, G, T; R-A, G; S-C,G; X-trinucleotides encoding 19 amino acids except cysteine

Table 5. BSA conjugated CTD peptides

Peptide	Sequence
CTD-BSA	Y S P T S P S Y S P T S P S C - BSA
S2-BSA	Y pS P T S P S Y S P T S P S C - BSA
S5-BSA	Y S P T pS P S Y S P T S P S C – BSA
S7-BSA	Y S P T S P pS Y S P T S P S C – BSA
S2S5-BSA	Y pS P T pS P S Y S P T S P S C - BSA
S2S7-BSA	Y pS P T S P pS Y S P T S P S C - BSA
S5S2-BSA	Y S P T pS P S Y pS P T S P S C – BSA
S5S7-BSA	Y S P T pS P pS Y S P T S P S C – BSA
S7S2-BSA	Y S P T S P pS Y pS P T S P S C - BSA
S7S5-BSA	Y S P T S P pS Y S P T pS P S C - BSA
S2S5S7-BSA	Y pS P T pS P pS Y S P T S P S C - BSA

RESULTS

Specific antibody repertoires in centenarians and its antigen

Finding the antibody repertoires using combinatorial peptide library

The sera of three populations; centenarians, healthy volunteers between age 60 and 79 (defined as old group), and healthy volunteers younger than 43 (defined as young group), were collected (Table 2). From the sera of centenarians, IgG fractions were purified by protein G column chromatography and used to enrich phages from the phage display of combinatorial peptide library through biopanning.

After the three rounds of biopanning, the phages preferentially reactive to the IgG pool of centenarians were selected by a phage enzyme immunoassay. Phage clones encoding two highly-homologous peptides were found repeatedly, of which sequences were ‘YSATLRY’ and ‘YSPTLFY’ (Figure 6). In enzyme immunoassay, these two peptides, either displayed on phage (Figure 6) or chemically synthesized and conjugated to bovine serum albumin (BSA) (Figure 7A), reacted with individual IgG fractions of centenarian with much higher frequency than IgG fractions of other group individuals. As each individual centenarian’s antibody titers to these two peptides were highly correlated. (Figure 7B).

Preparation of anti-YSATLRY polyclonal antibodies from human sera

In order to identify the antigen that reacts to the peptide, we needed larger quantity of the antibody. Since the quantity of sera from the centenarians are very limiting, we collected

sera from 59 healthy young volunteers to characterize and define the antigen represented by these two homologous peptides, and tested for enzyme immunoassay using peptides as an antigen (Figure 8).

Five volunteers showed significant antibody titers to both 'YSATLRY' and 'YSPTLFY' (Figure 8 ; volunteer 7, 11, 19, 47 and 50). Purifying by affinity column chromatography using a peptide-conjugated gel from these candidates, yielded five polyclonal antibodies (pAbs). The specificity of these pAbs was confirmed by competition enzyme immunoassay using YSATLRY peptide (Figure 9). The binding of pAbs to YSATLRYGGGSC-BSA conjugate coated on microtiter plate was hindered by YSATLRYGGGS in the soluble fraction.

Identification of antigen

Using the pAbs, we tried to identify the antigen. In immunoprecipitation, we found out that many human cell lines contained antigen reactive to these pAbs (Data not shown). We performed immunoprecipitate the antigen from human LoVo cell lysates, which provided the most prominent band in Coomassie staining. Immunoprecipitating LoVo cell lysates with pAbs prepared from volunteer #47 (pAb 47) and #19 (pAb 19), revealed three major protein bands. These protein bands were identified by mass spectrometry analysis (Figure 10A).

In mass spectrometry analysis, two proteins were identified as DNA-directed RNA polymerase II subunit RPB1 (RPB1, RNA polymerase II largest subunit, NP_000928) and DNA directed RNA polymerase II subunit RPB2 isoform 1 (NP_000929).

The identity of antigen was once more confirmed by immunoblot analysis using LoVo

cell lysate immunoprecipitates with five pAbs (Figure 11). As RPB1 CTD undergoes extensive phosphorylation, we used pan- (RPB1), phosphorylated Ser2 (pS2-RPB1) and Ser5 (S5-RPB1) -RPB1 antibodies to analyze the immunoprecipitates. As a result, we could observe that pAb 19, which showed higher signal in enzyme immunoassay with both YSATLRY and YSPTLFY (Figure 8), immunoprecipitated pan-, phosphorylated Ser2 and Ser5 RPB1 (Figure 11). It is most likely that not only pAb 19, but also others are reacting to phosphorylated specific RPB1, judging from the molecular weight of the immunoprecipitated product (Note the molecular weight shift than input band).

Generation of anti- YSATLRY monoclonal antibodies

Monoclonal antibodies (mAbs) generated from the B lymphocyte pool of volunteer #19. A phage display of combinatorial single chain variable fragment (scFv) library with a complexity of 1.84 X 10^9 was constructed using mRNA prepared from peripheral mononuclear cell fractions of volunteer #19 (pAb 19).

This human scFv library was constructed using overlap extension PCR of the VH and VL gene fragments (Figure 5). After four rounds of biopanning against YSATLRY peptide, scFv clones randomly selected showed reactivity to YSATLRY peptide in phage enzyme immunoassay (Data not shown). Sequence analysis revealed that two scFv clones were selected from library. These antibody clones were cloned into mammalian expression vector and expressed in HEK293F cells as scFv- humasn Fc fusion form.

Then the reactivity of two mAbs (scFv-human Fc fusion protein) was tested in enzyme immunoassay (Figure 12B). We synthesized YSATLRY-BSA, YSPTLFY-BSA as well as non-phospho CTD peptide (CTD-BSA), and with different phosphorylation pattern as

Ser5 and Ser2 phosphorylated CTD (pS5S2-BSA), and Ser5 phosphorylated CTD (pS5-BSA), and used as antigens (Figure 12A). The mAb 2 reacted to YSATLRY-BSA, YSPTLFY-BSA, pS5-BSA and pS5S2-BSA but not to CTD-BSA. This result confirmed that YSATLRY and YSPTLRY are mimicking phosphorylated CTD motif. The mAb 1 clone did not reacted to any of CTD peptides like pAb 19.

The reactivity of mAbs (scFv-human Fc fusion protein) to RPB1 was tested in immunoprecipitation. A band with molecular weight equivalent to RPB1 was visualized in the lanes loaded with mAb 1 and mAb 2 immunoprecpitates (Figure 13). And the identity of this protein bands were once more confirmed by immunoblot analysis (Figure 14). The antibody (RPB1) reactive to carboxy-terminal domain (CTD) of RPB1, YpSPTSPS (pS2-RPB1) and YSPTpSPS (pS5-RPB1) all reacted to the protein band (Figure 14).

Evaluating overall tendency to produce autoantibodies

To determine whether the subjects possessing IgG antibodies reactive to YSATLRY and YSPTLFY had an overall tendency to produce autoantibodies, we tested antinuclear antibody (ANA) levels in the sera of 45 centenarians and 25 old and 25 young volunteers. Of these, 4 centenarians and 1 old healthy volunteer showed detectable ANA levels. However, there was no correlation between ANA level and the levels of serum antibodies against YSATLRY and YSPTLFY (Figure 15).

Development of anti – phospho RPB1 CTD antibodies

Construction of synthetic antibody library

The synthetic scFv library was constructed using degenerate primers (Table 4) encoding the artificial sequence of HCDR3 on the backbone of trastuzumab (figure 16). The primers were designed to encode 7, 9, 10, 12, 14, 16 and 18 amino acid length HCDR3. All the amino acid sequence of V_L and V_H except HCDR3 was equivalent to that of trastuzumab. The sequence of linker between V_L and V_H was (Glycine–Glycine–Glycine–Glycine–Serine)$_3$. We constructed two different versions of HCDR3 sequence with amino acid length of 9 and 10 to accommodate more diversity. We also included sequences encompassing two cystines possibly to form a disulfide-constraint-loop inside HCDR3. The expected and achieved complexity were described in Figure 16.

Generation of anti-RPB1 CTD antibodies

We synthesized ten CTD peptides, including non-phosphorylated and differentially phosphorylated forms at serine residues, and used them as antigens (Table 5).

From five rounds of biopanning on ten phospho-YSPTSPSYSPTSPS-BSA conjugates, we enriched scFv clones and evaluated each clone's reactivity to the individual phosphopeptides and non-phosphorylated peptide control (data not shown) via phage enzyme immunoassay. After nucleotide sequencing of the clones, we selected a subset of clones showing specificity for serine phosphopeptides for further analysis. None of the clones carried mutations in the regions adopted from trastuzumab (Table 6).

Characterization of anti-RPB1 CTD antibodies

All the selected clones were expressed as a scFv-rabbit Fc fusion protein and their specificity was once more confirmed by enzyme immunoassay (Figure 17). Clone pCTD-1stS2 specifically reacted to peptides with phosphoserine at the second amino acid position of the first YSPTSPS repeat (Figure 17A), while clone pCTD-2ndS2 reacted to peptides with phosphoserine at the second amino acid residue of the second repeat (Figure 17A). Clone pCTD-1stS5 reacted to peptides with phosphoserine at the fifth amino acid residue of the first repeat (Figure 17B). Clone pCTD-S7S5 reacted to peptides with phosphoserine at the seventh position of the first repeat and the fifth position of the second repeat (Figure 17B). Clone pCTD-S7 reacted to peptides with phosphoserine at the seventh position of either the first or second repeat (Figure 17C). One clone, PanCTD, was reactive to all phospho- and non-phosphopeptides tested (Figure 17D).

To confirm the clones' reactivity to native RPB1 CTD, we performed immunoblots and found that all clones reacted to RPB1 CTD from HEK 293T cell lysates (Figure 18). Nearly every antibody showed high specificity to RPB1 CTD except pCTD-1stS2, which reacted nonspecifically with a 100-kDa protein. Although the reactivity of pCTD-S7S5 (0.05 μg/ml) to S7S5-BSA (Figure 17B) was higher than that of pCTD-S7 (0.05 μg/ml) to S7S5-BSA (Figure 17C), the intensity of the band in the immunoblot lane probed with pCTD-S7S5 (0.05 μg/ml) was lower than that probed with pCTD-S7 (0.01 μg/ml), which is expected considering the epitope of pCTD-S7S5 is less prevalent than that of pCTD-S7.

We also examined whether these antibodies could be used for ChIP analysis (Figure 19). Four clones failed to co-immunoprecipitate enough genomic DNA fragments to be analyzed by sequencing (data not shown). Clone pCTD-2ndS2 successfully

immunoprecipitated enough genomic DNA to be analyzed by sequencing. Interestingly, most of the DNA segments that were co-immunoprecipitated were distributed in the exonic regions of house-keeping genes such as ACTB and GAPDH. This particular pattern was very different from those achieved using pre-defined RPB1 panCTD or phosphorylated RPB1 CTD-Ser2 antibodies, which are available on public databases (panCTD: GSM935395, CTD-Ser2: GSM935383). Additionally, DNA fragments were confined to the exonic regions of specific isoforms in some genes, like HSPA8 and MYL9 (Figure 19B)

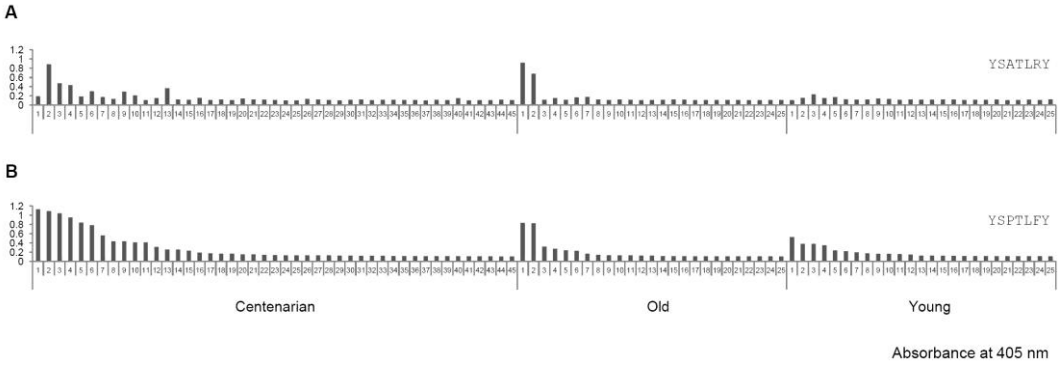

Figure 6. Reactivity of selected phage clones toward IgGs prepared from individuals of three groups.

Phages encoding YSATLRY (A) and YSATLFY (B) reacted to IgGs from centenarian group with much higher frequency than other two groups. A. About 10% of centenarians reacted to the YSATLRY peptide. B. About 25% of centenarians reacted to YSATLFY while reaction rate was 8% and 4% in old and young group, respectively. Many individual sera, which reacted to YSATLRY also reacted to YSATLFY.

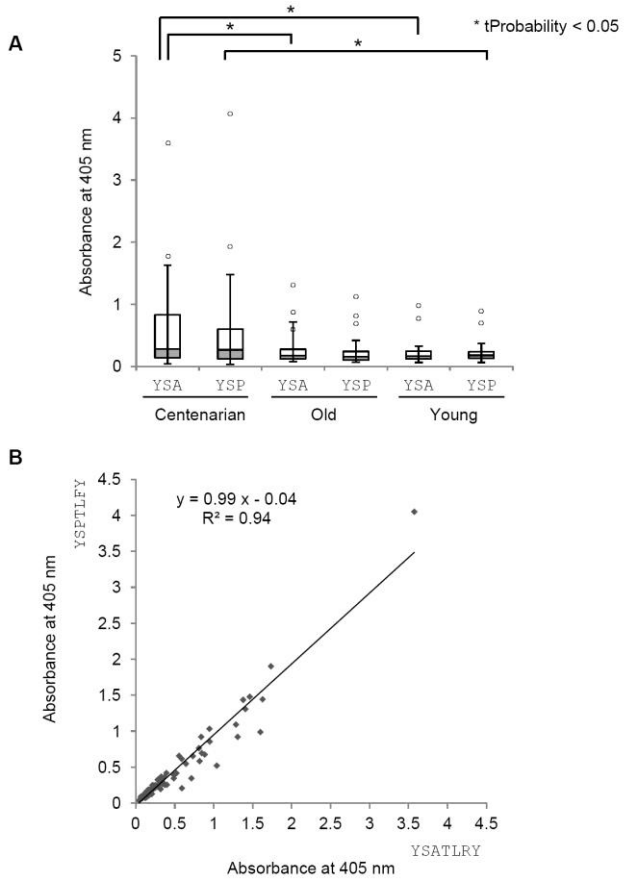

Figure 7. Corealations between YSATLRY (A) and YSPTLFY (B).

A. YSATLRY and YSPTLFY peptides were synthesized and conjugated to BSA and the reactivity to the individual sera of three groups was determined by enzyme immunoassay. The positive rate to YSATLRY was significantly different in three groups. The positive rate to YSPTLFY was significant higher in centenarian than young group. B. The enzyme immunoassay values were determined by individual sera toward YSATLRY and YSPTLFY, showed strong correlation (R = 0.94). This result indicates that these two peptides are actually representing the same epitope.

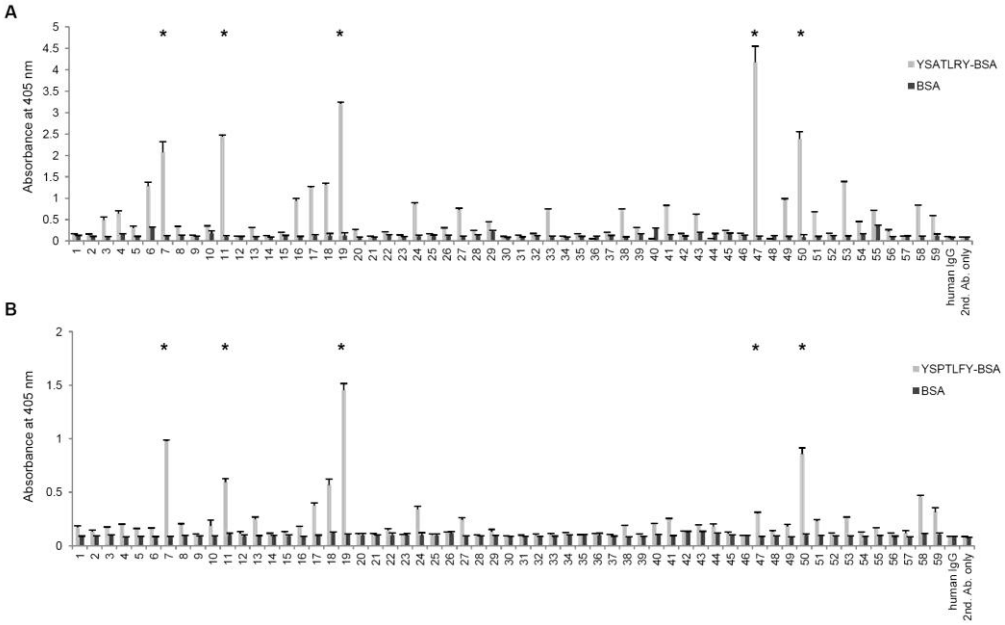

Figure 8. Enzyme immunoassay to determine antibody titer to YSATLRY (A) and YSPTLFY (B).

The wells of the microtiter plate were coated with BSA conjugated peptide 'YSATLRY' or 'YSPTLFY'. After blocking, individual human sera was incubated and washed. Afterwards, plate was incubated with a HRP conjugated anti-human IgG antibody and the amount of bound antibody was determined using ABTS as a substrate. Asterisks indicate selected clones that have positive signals.

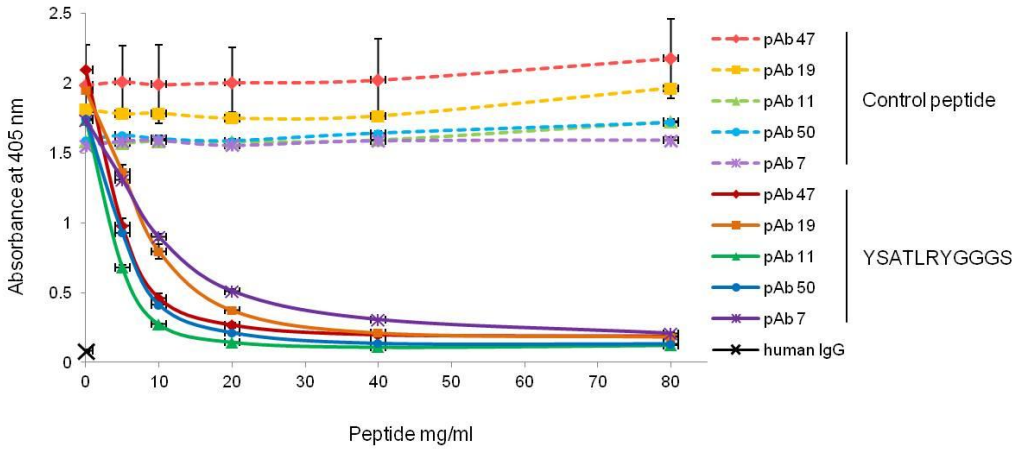

Figure 9. Competition enzyme immunoassay using polyclonal antibodies with peptide YSATLRY.

Columns were cross linked with peptide 'YSATLRY', and anti-peptide 'YSATLRY' polyclonal antibodies were purified from human sera. Purified antibodies were subjected to competition ELISA to test the affinity to the peptide. The wells of the microtiter plate were coated with BSA conjugated peptide. Purified anti-peptide 'YSATLRY' polyclonal antibodies were pre-incubated with indicated concentrations of peptide. After blocking procedure, pre-incubated antibodies were added to the plate. Then the plate was washed and incubated with HRP conjugated anti-human IgG antibody. The amount of bound antibody was determined using ABTS as a substrate. LPCYTDHICYSSGGGS was used as a control peptide.

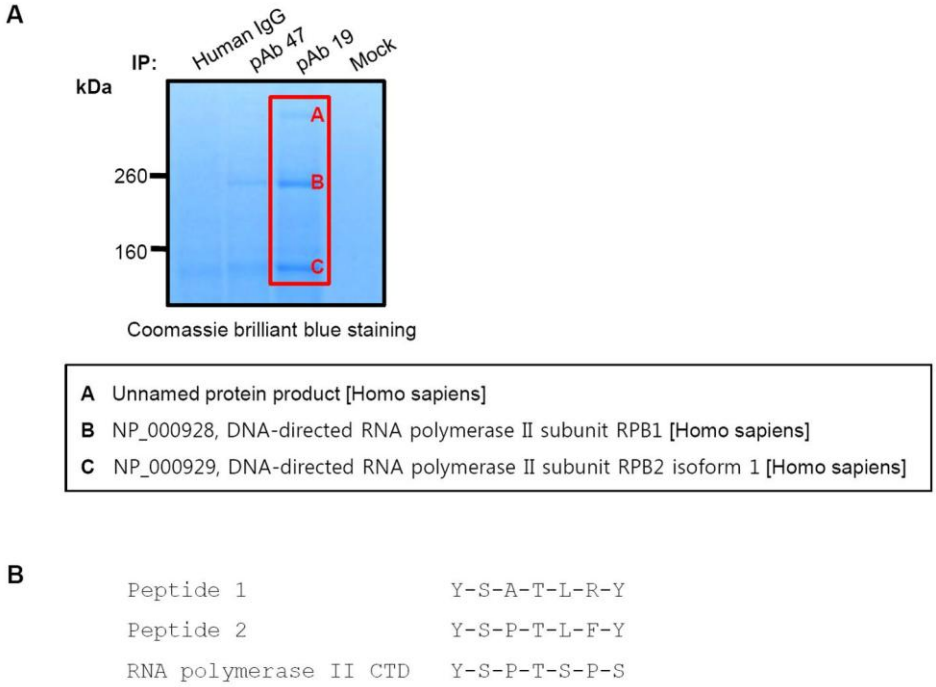

Figure 10. Identification of RPB1 CTD as the antigen selection to centenarian's IgG. A. LoVo cell lysates were immunoprecipitated with polyclonal antibodies against YSATLRY. Commassie brilliant blue staining of immunoprecipitates. 3 candidate bands (marked as A, B and C), precipitated with polyclonal antibody #19 were subjected to mass spectrometry analysis. B. Amino acid sequence comparison of two peptide which showed strong affinity to IgG of centenarians and heptapeptide repeat sequence of RPB1 CTD.

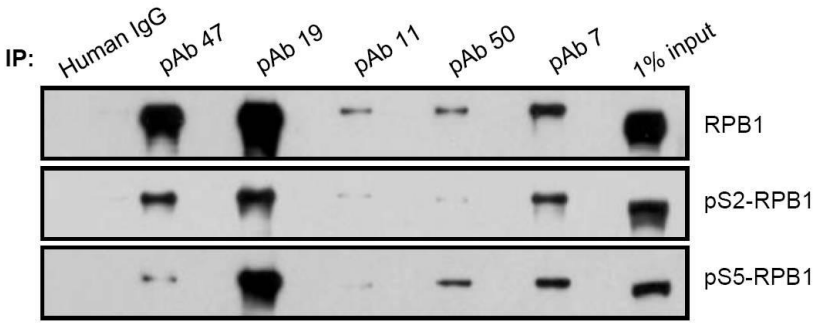

Figure 11. Immunoprecipitation and immunoblot with polyclonal antibodies.

Immunoprecipitates from LoVo cell lysates using polyclonal antibodies, were probed for immunoblot with anti-pan (RPB1), phosphorylated Ser2 (pS2-RPB1), and Ser5 (pS5-RPB1) RPB1 CTD antibody.

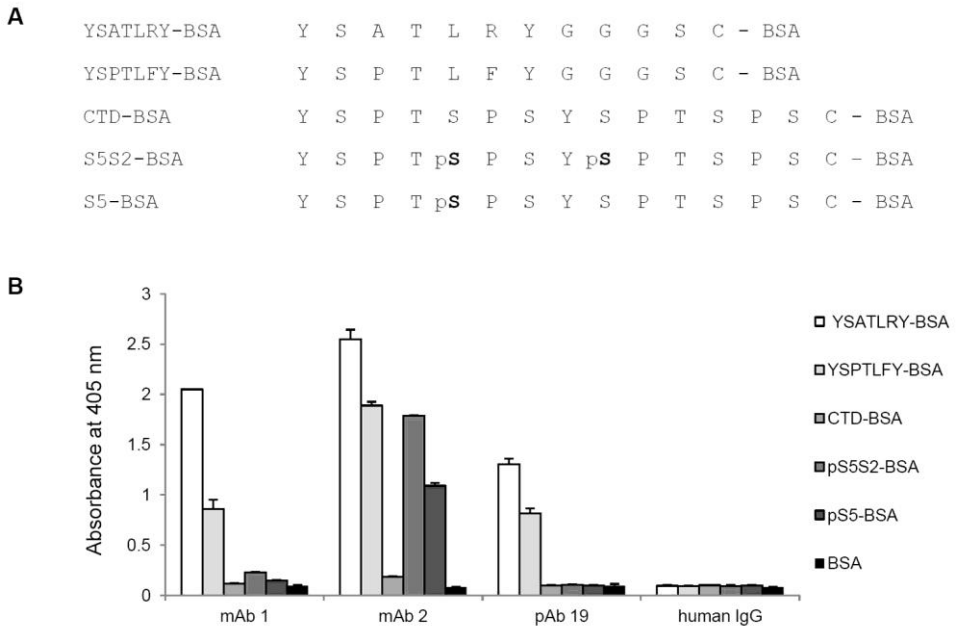

Figure 12. Two monoclonal antibodies (scFv-Fc fusion peotein) generated from human naïve library.

A. Sequence of peptides used in (B) were indicated. B. Monoclonal antibodies were constructed as a fusion form of human scFv (single chain fragment variable region) and human Fc. Purified antibodies were probed for enzyme immunoassay with indicated peptides. Phosphorylated forms of RPB1 CTD were generated as BSA conjugated peptide and coated on the wells of the microtiter plate. After blocking, mAb 1, mAb 2, pAb 19 and human IgG were added to the wells. Plate was incubated with HRP conjugated anti-human Fc specific antibody and signals were detected using ABTS solution.

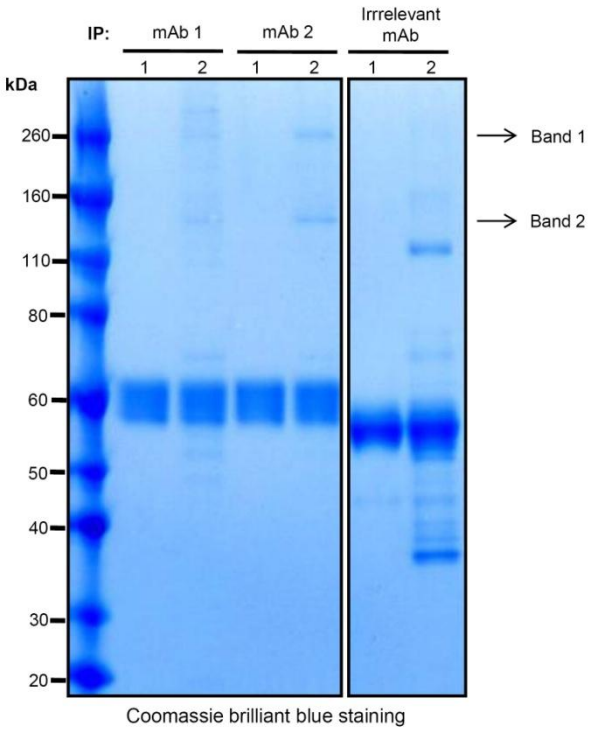

Figure 13. Immunoprecipitation of LoVo cell lysates with monoclonal antibodies.

LoVo cell lysates were incubated with either mAb 1 or mAb 2 (as scFv-human Fc fusion protein) conjugated to protein A beads. After washing, the immunoprecipitate was subjected to NuPage 4–12% Bis-Tris gel electrophoresis (Lane 2). The gel was stained with Coomassie Brilliant Blue. Irrelevant scFv-human Fc fusion protein (irrelevant mAb) was used as a control in immunoprecipitation experiments. Lane 1 was loaded with the eluate of the mAb-conjugated gel without incubation with LoVo cell lysates. Bands 1 and 2 were expected to be RPB1 and RPB2 isoform 1, respectively.

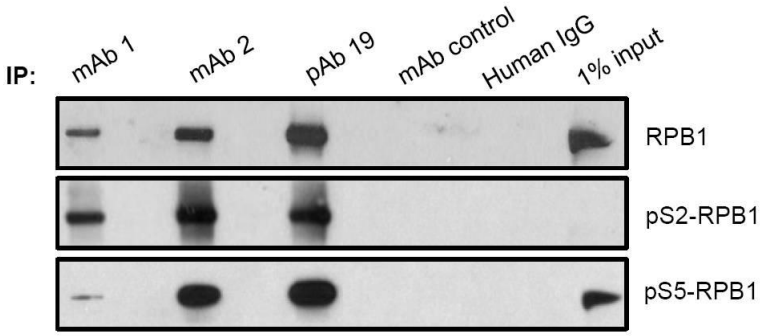

Figure 14. Immunoprecipitation and immunoblot with monoclonal antibodies.

Two monoclonal antibodies (mAb 1 and mAb 2) were generated through biopanning on peptide 'YSATLRY', applied on immunoprecipitation using LoVo cell lysates. Immunoprecipitates were probed for immunoblot with anti-pan, phosphorylated serine 2 and serine 5 antibodies. Irrelevant antibody (mAb control) and human IgG were used as a negative control.

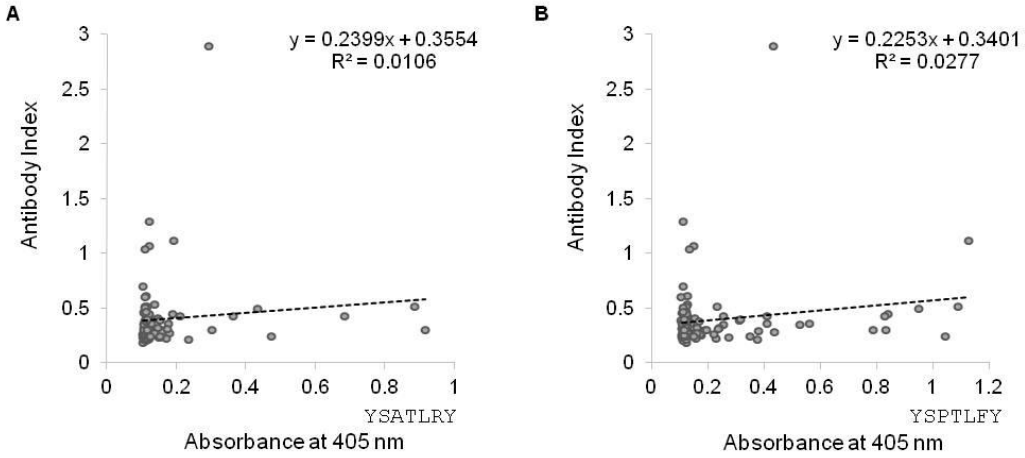

Figure 15. Correlation of ANA antibody index with antibody reactivity to YSATLRY (A) and YSPTLFY (B).

The sera of 45 centenarian, 25 old, and 25 young volunteers were tested for ANA using an enzyme immunoassay kit. Optical densities were converted to antibody index according to the manufacturer's instructions.

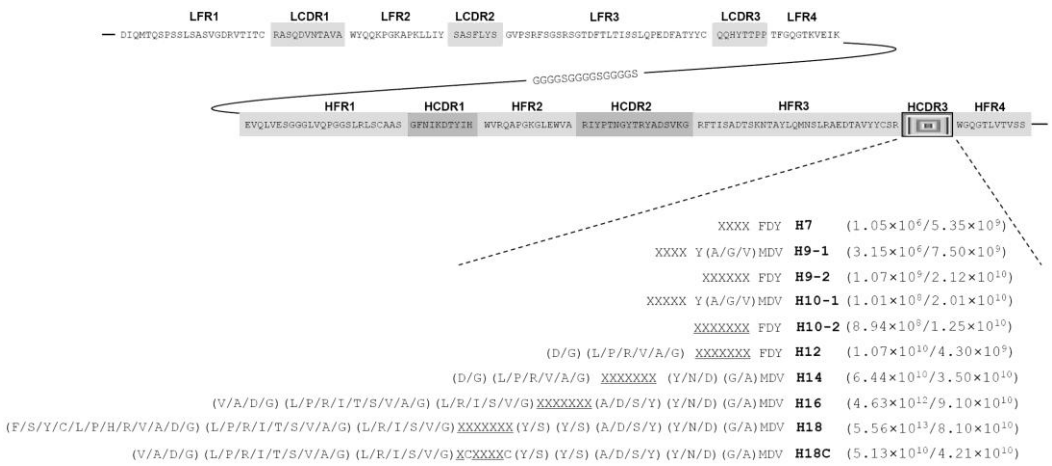

Figure 16. Sequences of constructed synthetic antibody libraries.

The amino acid sequence of the scFv, except HCDR3, was adopted from trastuzumab. The length of HCDR3 varies from seven to eighteen amino acids. The H18C library contains two cysteines to form an intra-HCDR3 disulfide bond. “X” represents residues randomized by NNK nucleotide sequence, and “<u>X</u>” (underlined) represents residues randomized by a mixture of trinucleotides encoding 19 amino acids except cysteine. When a position is diversified with less than 19 amino acids by partially degenerate codons, the encoded amino acids are shown inside parentheses. The complexity required to cover all possible sequences and the actual complexities achieved in this study are shown in parentheses (expected complexity/achieved complexity).

Table 6. HCDR3 amino acid sequences of antibody clones

Phosphopeptides used in biopanning	Clone	HCDR3 sequence (amino acid length)
S2-BSA	pCTD-1stS2	GAFWFSRSHFDY (12)
S2S5-BSA	pCTD-1stS5	GPWPGSHHKYAMDV (14)
S2S5S7-BSA	pCTD-S7	GISYPWQRGYYYAMDV (16)
S5S2-BSA	pCTD-2ndS2	DRAQWWEYKNAMDV (14)
S7S5-BSA	pCTD-S7S5	DPWSRWAKVFDY (12)
S2-BSA	panCTD	YGRRGVFDY (9)

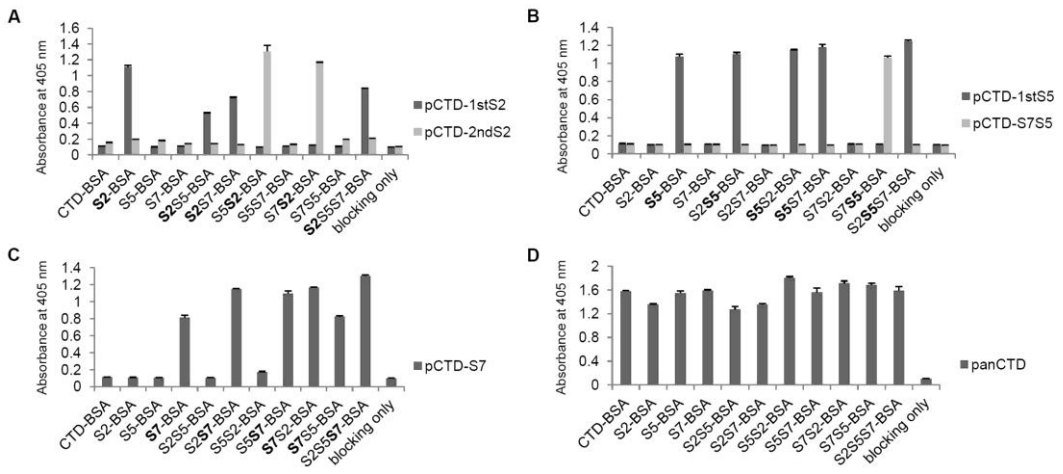

Figure 17. Reactivity of selected antibodies to BSA conjugated CTD peptides.

The each well of microtiter plate was coated with BSA conjugated CTD peptides. The scFv-Fc fusion protein was added to each well at the concentration of 0.05 μg/ml for pCTD-1S2 (A), pCTD-1S5 (B), pCTD-S7 (C) and pCTD-S7S5 (B) or 1 μg/ml for pCTD-2S2 (A) and CTD-pan (D). The plate was washed and incubated with anti-rabbit IgG Fc antibody - HRP conjugate. After washing, the amount of bound antibody was determined using ABTS as a substrate.

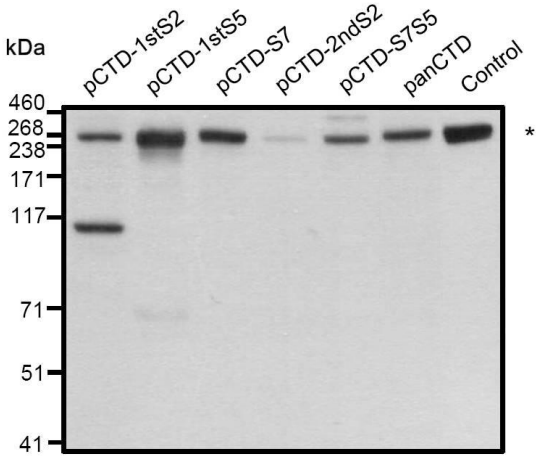

Figure 18. Reactivity of antibody clones to RPB1 CTD in immunoblot analysis.

HEK 293T cell lysate was subjected to gel electrophoresis. After electrophoresis, the protein in the gel was transferred to NC membrane. After blocking, the membrane was probed with scFv-Fc fusion proteins. The location of bound scFv-Fc fusion protein was visualized using HRP conjugated anti-rabbit IgG Fc antibody and SuperSignal Pico West chemiluminescent as a substrate. A commercial phosphorylated S2 CTD antibody was used as a positive control. Asterisk indicates the expected location of RPB1 CTD.

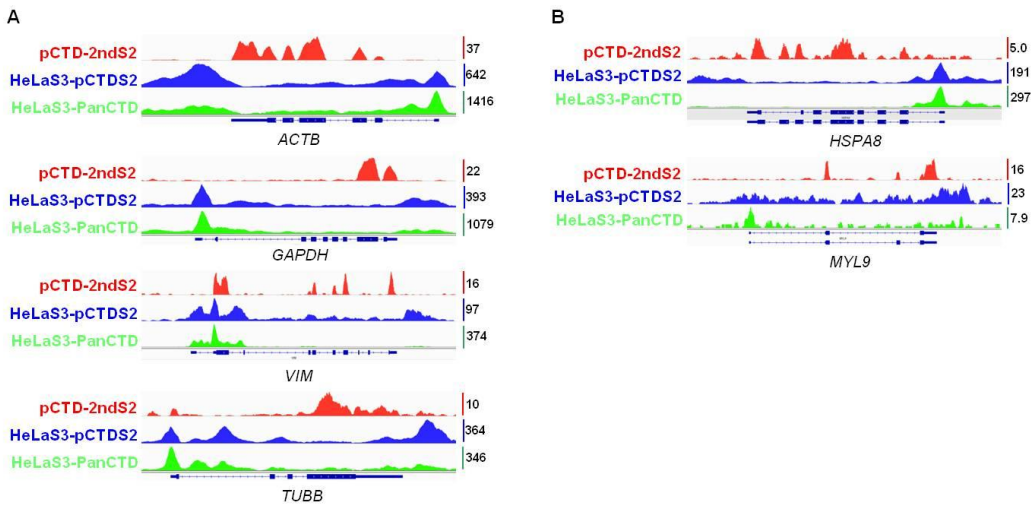

Figure 19. ChIP binding profiles.

pCTD-2ndS2 ChIP results, showing an exonic pattern of distribution, were compared to public data (phosphorylated S2, HeLaS3-pCTDS2; panCTD, HeLaS3-PanCTD) (A and B). B. pCTD-2ndS2 profiles showing isoform-specific patterns. Scale data ranges are indicated on the right side of each individual track. RefSeq gene structures are depicted below the profiles.

DISCUSSION

As it became more and more of an aging society, the interest of living healthy in one's advanced age is growing nowadays. In this aspect, importance of centenarian research has shed a new light [8]. They not only maintain a longer lifespan than normal person, but also are resistant to senile disease and harbor both healthy physical and mental status. In this study, we hypothesized that centenarian specific antibody could be resided in the serum, and the antibody has been generated to make the host live longer in the process of longevity.

For this purpose, we used phage display technology, which enables to find specific antigen from certain group of antibodies, to investigate the antigen of centenarian specific antibody. Initially, we selected the peptides that bind specifically to the antibody of centenarians (Figure 6). Afterward, the protein, which the peptides mimic, was identified via proteomic methodology. Selected two peptides, which specifically reactive to centenarians' IgG, were sequenced as 'YSATLRY' and 'YSPTLFY'.

These peptides, which showed higher reactivity to centenarians, were selected by comparing different aged groups (old, young). More than 10, out of 45 centenarians, that is, about 25% were positive to these two peptides (Figure 7A). This means that it is statistically significant than the result - about 8% of old and 4% of young individuals showed positive reactivity to the peptide. From the fact that each individual centenarian's antibody titers to these two peptides were highly correlated (Figure 7B), we assumed that these two peptides might represent the same epitope of the antigen. By mass spectrometry

analysis, followed by immunoprecipitation, we have identified the antigen.

To our surprise, two proteins were identified as DNA-directed RNA polymerase II subunit RPB1 (RPB1, RNA polymerase II largest subunit, NP_000928) and DNA directed RNA polymerase II subunit RPB2 isoform 1 (NP_000929) (Figure 10A). As both proteins are components of RNA polymerase II complex [70], we concluded that the pAbs immunoprecipated a part of RNA polymerase II complex. These pAbs reacted to the protein with molecular weight similar to RPB1 (Figure 10A), we searched the amino acid sequences homologous to YSATLRY and YSPTLFY inside RPB1. We found out that the carboxy-terminal domain (CTD) of RPB1 is composed of multiple repeat of YSPTSPS that is significantly homologous to YSATLRY and YSPTLFY (Figure 10B).

We confirmed that pAbs could precipitate hyperphosphorylated CTD by immunoprecipitation-immunoblot using commercial antibodies reactive to YpSPTSPS (pS2-RPB1) and YSPTpSPS (pS5-RPB1) (Figure 11).

For establishing the stable source of the antibody, a phage display of combinatorial single chain variable fragment (scFv) library was constructed using mRNA prepared from peripheral mononuclear cell fractions of volunteer #19, possessing the antibody to the epitope. Through biopanning on YSATLRY, two clones reactive to both of the peptides (YSATLRY and YSPTLFY) and the antigen were successfully selected (Figure 12). Using these mAbs, once more confirmed that, in good agreement with the data from Figure 14, mAb 2 was indeed more specific to phosphorylated RPB1 CTD.

Eventually, we revealed that YSATLRY and YSPTLRY mimicking the phosphorylated form of YSPTSPS (CTD of RPB1). However, the repetitive nature of the CTD makes it hard to determine exactly which of the 52 heptads (in mammals) are phosphorylated.

What's more, it was not easy to clarify which form of phosphorylated CTD, YSATLRY and YSPTLFY represent. To make it work, various antibodies that precisely recognize each phosphorylated form of CTD is needed.

As a matter of fact, there are already numerous CTD antibodies which are commercially available and some antibodies reported to the serine-phosphorylation pattern of CTD [21]. However, none of these products are humanized antibodies. Also their specificity has been only minimally characterized, and their amino acid sequences are not yet publically available. With this circumstance, we conclude that the pre-existing antibodies for phosphorylated CTD were insufficient for reflecting the delicate and dynamic nature of CTD phosphorylation.

For these reasons, we constructed a synthetic antibody library with HCD3-confined diversity and selected antibodies recognizing serine phosphorylation patterns of RPB1 CTD. Antibodies against post-translational modifications of protein are difficult to generate in higher affinity form. Especially, in the generation of antibody to phosphorylated protein motif, synthetic antibody library is valuable as phosphorylated peptide antigens often fail to produce specific antibodies from antibody library of either immunized or naïve repertoire [47].

And we used synthesized various phosphorylated-CTD peptides as antigens (Table 4) for development of antibodies. As previously reported, a twice-repeated sequence of YSPTSPS or YSPTSPSYSPTSPS is a functional unit and has an optimal amino acid length for epitope functionality [71] that could facilitate the use of these developed antibodies for functional assays. Because CTD has a flexible three-dimensional structure [72], the domain can accommodate structural changes necessary for binding with a

synthetic antibody.

Considering the diverse patterns of RPB1 CTD phosphorylation and its functional role in determining interactions with binding proteins, antibodies specific to a certain pattern of CTD serine phosphorylation are valuable tools for studying the physiological role of CTD phosphorylation.

When screening antibody clones, we attempted to isolate antibodies that could discriminate specific CTD phosphorylation patterns but realized this type of antibodies are scarce (data not shown). We identified only one clone (pCTD-S7S5) specific to the S7S5 peptide but not reactive to the other nine phosphopeptides and non-phosphopeptide control (Figure 17). All other clones reacted to more than one phosphopeptide. Biopanning on peptides with multiple phosphoserine residue resulted in clones with broader specificity to phosphopeptides other than those individually used in biopanning (Table 6). These data also strongly support that high-throughput screening of antibody sequences using next-generation sequencing (NGS) would be required to select rare antibody clones specific to peptides with multiple phosphoserines in future studies [59], [60]. The library we constructed in this study is optimal for this purpose because only nucleotide sequencing of HCDR3 is required, and the scFv gene can be easily synthesized using nucleotide sequence information obtained from NGS. Alternatively, developing antibodies with more stringent specificity may be possible by generating daughter libraries with randomized key amino acids in other CDRs [73].

We selected five antibodies showing different specificity based on the phosphorylated patterns of CTD in the enzyme immunoassay (Figure 17) for further analysis. All of these antibodies successfully reacted to RPB1 CTD in immunoblot analysis, which proved that

their reactivity is not limited to phosphopeptides. We also tested whether these antibodies can be used for ChIP analysis, which was quite challenging as there is only a limited number of monoclonal antibodies available for ChIP analysis. It is well known that monoclonal antibodies have a disadvantage in ChIP experiments because they only recognize a single epitope, and therefore there is a higher likelihood that the epitope will be masked by transcription factors or genomic DNA [74]. Consistent with these challenges, four antibody clones failed to co-immunoprecipitate enough DNA material to be analyzed by sequencing. However, clone pCTD-2ndS2 successfully immunoprecipitated RPB1 that interacted with genomic segments confined to exonic regions (Figure 19). To our knowledge, no RPB1 CTD phosphorylation pattern has been reported to mark exonic regions. It is well known that DNA segments co-immunoprecipitated with RPB1 panCTD antibody show a widely distributed pattern throughout genomic loci, with a greater abundance at the transcription start site. RPB1 CTD phosphorylated at Ser2 shows another distribution pattern, predominantly interacting with 3′ of transcription termination sites of genes, where it can recruit transcription termination factors [75].

The messenger RNA processing reactions of capping, splicing, and polyadenylation are believed to occur co-transcriptionally. The transcribed pre-mRNA itself contains several consensus elements in *cis* that are essential for the splicing reaction [76]. Therefore, it is a reasonable assumption that RPB1 CTD with a certain phosphorylation code may recruit mRNA processing factors to facilitate this reaction. Because exonic regions are marked with cross-exon recognition complexes that contain serine/arginine-rich (SR) proteins [77], and SR proteins are known to interact with phosphorylated RPB1 CTD, it would be

interesting to determine whether pCTD-2ndS2 phosphorylation in the double heptamer CTD unit is a code for SR protein recruitment. In addition, mammalian splicing factor Spt6 is reported to interact selectively with phosphorylated S2 through its SH2 domain and mediate hlws1-dependent splicing [78]. However, Spt6 does not bind to a single CTD repeat [79], suggesting that a pattern of multiple phosphorylated sites over multiple heptamer repeats create a docking platform for such a protein. It is possible that our pCTD-2ndS2 antibody is reacting to such a modification on the chromatin, as seen in our genome-wide ChIP-seq data (Figure 19).

As a conclusion, we found out that centenarians possess IgG antibody reactive to YSATLRY and YSPTLRY, mimicking the phosphorylated form of YSPTSPS motif in much higher frequency than average population.

However, several questions still remain. Why does 'the antibody (centenarian specific)' detect RPB1 CTD? Is it the cause or consequence of the longevity?

RNA polymerase was reported to function as autoantigen in scleroderma patients [80], [81], [82], [83]. We tested antinuclear antibody (ANA) levels in the sera of 45 centenarians and 25 old and 25 young volunteers to check whether the subjects possessing IgG antibodies reactive to YSATLRY and YSPTLFY had an overall tendency to produce autoantibodies. As a result, there was no correlation between ANA level and the levels of serum antibodies against these peptides (Figure 15).

It was suggested that the repetitive amino acid sequence and high content of charged residues of the CTD structure contribute to its role as an autoantigen [84]. But the life expectancy of the patients with scleroderma is not longer or shorter than healthy

individual [85]. At this moment, it is not clear whether the auto-antibody is providing protective function to centenarians. The anti-phospho RPB1 CTD antibodies might be results of aging process for centenarians to be exposed for longer period of time than average population. To the limit of our knowledge, this is the first report about the presence of auto-reactive antibodies in centenarians with a higher frequency than average population. The mechanism how these antibodies were generated and the role of these antibodies in human (patho) physiology would be an interesting topic for us to follow.

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국문 초록

적응 면역은 개체를 외부의 공격으로부터 보호해주는 자기 방어 시스템의 하나로, 체내에 침입한 세균, 바이러스, 암과 같은 공격원에 대한 효과적인 특이적 방어수단을 갖게 하여 개체의 생존에 유리하게 작용한다. 본 연구에서는 지금까지 밝혀지지 않은 백세인에게 특이적으로 존재하는 항체가 그들의 장수에 어떠한 기여를 하였는지 알아보기 위하여, 최근 도입된 phage display 기법을 이용하여 백세인의 적응면역을 분석해 보고자 하였다. 즉, 백세인에게 특이적으로 존재하는 항체 repertoire 가 존재하는지 알아보기로 하였다.

실험 대상자를 백세인과 대조군 그룹으로 나눈 후, 각 그룹의 혈청으로부터 IgG 를 분리하였다. 그 다음, combinatorial peptide library 와 phage display 기법을 이용하여 백세인의 IgG 에 특이적으로 결합하는 펩타이드를 선별하였고, 두 개의 펩타이드가 다른 대조군에 비해 백세인에게 높은 반응성을 보이는 것을 확인하였다. 펩타이드 서열을 분석한 결과, YSATLRY 와 YSPTLFY 로 서열의 상동성이 높은 펩타이드로 밝혀졌다. 우리는 이 두 펩타이드를 소 혈청 알부민 접합 형태로 화학적 합성 하였고, 효소면역분석법을 이용하여 다른 대조군 그룹에 비해 백세인에게 높은 반응성을 보이는 것을 재확인 하였다. 또한, 두 펩타이드에 대한 항체 역가가 높은 수치로 일치하는 것을 확인함으로써, 이 두 가지 펩타이드는 같은 항원결정부위를 대리할 것으

로 예상하였다.

이 두 펩타이드가 대리하는 항원을 규명하기 위하여, 지원자를 새로 모집하였고, 그들의 혈청으로부터 YSATLRY 에 대한 다중클론성 항체를 정제하였다. 정제된 항체로 대장암 세포주인 LoVo 세포 용해물을 면역침전시키고, 이로부터 침전된 단백질을 얻어 질량분석법으로 동정하였다. 해당 단백질은 'DNA-directed RNA polymerase II subunit RPB1 (RPB1)' 로 밝혀졌다.

규명된 항원의 재확인을 위해, 판매되는 항체를 이용한 면역블롯방법을 수행하였다. 더 안정된 항체의 확립을 위해, 우리는 지원자의 인체 말초 단핵세포로부터 mRNA 를 얻어 항체 라이브러리를 제작하였다. 그리고, YSATLRY 에 대한 바이오패닝을 수행하여, YSATLRY 와 YSPTLFY 두 펩타이드에 모두 반응성을 갖는 단클론항체를 성공적으로 선별하였다. 특히, 한 단클론항체는 RPB1 의 카르복시 말단 (CTD) 의 인산화된 형태에 반응성을 갖는 것으로 확인되었다.

RPB1 CTD 의 인산화된 형태를 더욱 명확히 규명하기 위하여, 다양한 형태의 인산화 CTD 에 결합하는 항체를 제작하였다. 항체 제작을 위하여, 우리는 항 HER2-항체의 HCDR3 부분이 7-18 아미노산 잔기로 대체된 '합성 항체 라이브러리'를 제작하였다. 항체는, CTD 반복서열의 일반적인 기능단위인 '$(YSPTSPS)_2$'의 인산화 형태의 펩타이드에 대하여 선별되었다. 항체 클론 pCTD-1stS2 와 pCTD-2ndS2 는 CTD의 일곱개의 반복아미노산 서열 (YSPTSPS) 중, 두 번째 Serine 이 인산화되어 있는 펩타이드에 반응성을 갖

는 클론으로 확인되었다. pCTD-1stS5 클론은 다섯 번째 Serine 이 인산화되어 있는 형태의 펩타이드에 반응성을 가졌고, pCTD-S7S5 는 일곱 번째 Serine 과 그 뒤에 다섯 번째 Serine 이 인산화되어 있는 펩타이드에만 반응성을 가졌다. 마지막으로 pCTD-S7 클론은 일곱 번째 Serine 이 동시에 인산화되어 있는 펩타이드에는 모두 반응성을 갖는 클론으로 확인되었다. 이 모든 항체는 면역블롯방법으로 세포 내 RPB1 CTD 에 잘 반응하는 것을 확인하였다. 흥미롭게도, 항체 클론 pCTD-2ndS2 는 전-유전체 크로마틴 면역침강 서열 분석방법에서 유전자의 엑손 영역에 존재하는 RNA 중합효소 II 를 두드러지게 침강시켰다. 이것은 기능단위 $(YSPTSPS)_2$ 의 두 번째 반복서열 중 두 번째 인산화 Serine 이 엑손 결정의 매개체인 것을 시사한다.

결론적으로, 일반 사람보다 백세인에게 특이적으로 존재하는 항체가 있음을 확인하였고, 그 항원이 RPB1 임을 규명하였다. 또한, 이 항원 (RPB1)의 다양한 인산화 형태에 대한 항체를 제작하였다.

주요어 : 노화, 백세인, phage display, RNA 중합효소 II, RPB1, CTD, 인산화

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