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Optimized Serological Isolation of Lung-Cancer-associated Antigens from a Yeast Surface-expressed cDNA Library

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Abstract The technique of serological analysis of antigens by recombinant cDNA expression library (SEREX) uses autologous patient sera as a screening probe to isolate tumor-associated antigens for various tumor types. Isolation of tumor-associated antigens that are specifically reactive with patient sera, but not with normal sera, is important to avoid false-positive and autoimmunogenic antigens for the cancer immunotherapy. Here, we describe a selection methodology to isolate patient sera-specific antigens from a yeast surface-expressed cDNA library constructed from 15 patient lung tissues with non-small cell lung cancer (NSCLC). Several rounds of positive selection using patient sera alone as a screening probe isolated clones exhibiting comparable reactivity with both patient and normal sera. However, the combination of negative selection with allogeneic normal sera to remove antigens reactive with normal sera and subsequent positive selection with patient sera efficiently enriched patient sera-specific antigens. Using the selection methodology described here, we isolated 3 known and 5 unknown proteins, which have not been isolated previously, but and potentially associated with NSCLC.

Keywords: SEREX, yeast surface display, cDNA library, tumor-associated antigens, non-small cell lung cancer

The identification of tumor-associated antigens that are overexpressed or specifically expressed in tumors is a cornerstone for the development of cancer diagnostic, prognostic, and therapeutic strategies. One of the powerful approaches to identify tumor-associated antigens is serological analysis of antigens by recombinant cDNA expression library (SEREX) [14, 17]. Tumors are caused by mutations in the DNA of cells, many of which would alter expression properties of the encoded proteins. Aberrantly expressed proteins are often immunogenic to elicit the humoral and/or cellular immune responses, generating circulating immunoglobulin G antibodies that specifically recognize the tumor-associated antigens in an autologous host [4, 17]. Based on the spontaneous humoral immune response, SEREX uses autologous patient sera as a screening probe to isolate tumor-associated antigens from a cDNA expression library generated from specific tumor cells and/or tissues [14, 17]. SEREX has been used successfully to identify a variety of new tumor-associated antigens in many different tumor types [5–7, 17], with more than 2,000 antigens deposited in the SEREX database [16].

For the expression of cDNA library, SEREX has extensively adopted a prokaryotic expression system, such as *Escherichia coli* and phage display, in which ELISA, Western blotting, and/or biopanning are used as high throughput screening techniques. However, the bacterial expression system has an intrinsic limit in that eukaryotic proteins might not be folded correctly in prokaryotes because of lack of proper chaperones and posttranslational modifications [2, 15, 16]. Posttranslational modification of proteins could also cause immunogenicity by generating neoantigenic epitopes [4]. Thus, SEREX using prokaryotic expression systems could not isolate tumor-associated antigens that elicit antibodies recognizing their conformationally or posttranslationally modified epitopes [15, 16, 22]. To overcome the limits of prokaryotic expression of a cDNA library, very recently, a yeast surface display system has been employed for the surface expression of a cDNA library from breast cancer tissues and subsequent serological isolation of breast cancer-related antigens [22]. SEREX using a yeast surface display system enlarges the potential spectrum of antigens detected by patient sera [15, 22, 23].

Identification of tumor-specific antigens that are selectively detected by patient sera, but not by normal sera,

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is important for successful immunotherapeutic approaches to cancer by avoiding autoimmune diseases [9]. To isolate cancer-specific antigens during SEREX, a cDNA library was constructed after the testicular cDNA library was subtracted with mRNA from normal healthy tissues [19]. However, there have been no reports describing a selection strategy to isolate patient sera-specific antigens. Conventional SEREX employs autologous patient sera alone, but not normal sera, in the selection procedures [5–7, 17, 22]. In this study, we describe an efficient selection strategy to isolate patient sera-specific antigens from a yeast surface-expressed cDNA library constructed from 15 patient lung tissues with non-small cell lung cancer (NSCLC). We introduced negative selection procedures, where allogeneic normal sera from healthy donors were probed to remove antigens reactive with normal sera, and then carried out positive selections with autologous patient sera. We found that, compared with only positive selections with patient sera, the combination of negative and positive selections efficiently isolated patient sera-specific antigens that have not been isolated previously.

MATERIALS AND METHODS

Materials

Anti-c-myc monoclonal antibody (mAb) 9E10 was purchased from Ig Therapy (Chunchun, Korea). Bovine serum albumin (BSA), FITC-conjugated goat anti-human IgG, and R-phycoerythrin-conjugated goat anti-mouse IgG were from Sigma. All other chemicals and solvents used were of analytical grade. The oligodeoxynucleotides were synthesized from Bionics Co. (Seoul, Korea). The yeast *Saccharomyces cerevisiae* EBY100 strain and the yeast surface display plasmid, pCTCON, have been previously described in detail [3, 11, 12].

Patients

The detailed clinical profiles of 15 patients with NSCLC are described in Table 1. Histologic types of the NSCLC samples consisted of 8 squamous cell carcinomas, 6 adenocarcinomas, and 1 large cell carcinoma, and the pathologic stages were classified according to the guideline of the American Joint Committee on Cancer (AJCC) (Table 1). After informed consent, tissue specimens and blood samples from 15 patients with NSCLC and blood samples from 8 healthy donors (7 ml per individual) were collected under permit of the Ethical Committee of the Samsung Medical Center. Patient blood was obtained preoperatively in the operating room. Serum, which is free of cells and clotting factors, was prepared by using the SST II Advance 8.5-ml tube (BD Vacutainer Systems, NJ, U.S.A.) according to the user manual and stored at −70°C until used. Tumor tissues were collected and prepared for RNA isolation in fresh state just immediately after resection of lung cancer in consecutive operation schedules.

Construction of cDNA Library from Lung Cancer Tissues

Individual total cellular RNA was isolated from tumor tissues of 15 patients with NSCLC using TRIzol reagent (Invitrogen) according to the recommended protocol. Isolation of mRNA from the respective total RNA was carried out using an Oligotex mRNA isolation kit (Qiagen) and pooled together for cDNA library construction. The cDNA library was constructed using a SMART cDNA Library Construction Kit (Clontech, Germany) according to the manufacturer's instructions, except that the primers

Table 1. Clinical characteristics of 15 patients with non-small cell lung cancer.

Case	Sex	Age (years)	Histology	AJCC pathologic stage[a]
1	M	57	Squamous cell carcinoma	IIA
2	M	71	Squamous cell carcinoma	IIIA
3	F	65	Adenocarcinoma	IA
4	F	65	Adenocarcinoma	IIB
5	F	72	Adenocarcinoma	IB
6	M	62	Adenocarcinoma	IIIA
7	M	61	Squamous cell carcinoma	IA
8	M	67	Squamous cell carcinoma	IIB
9	M	67	Sarcomatoid carcinoma	IIIA
10	M	69	Squamous cell carcinoma	IIB
11	F	51	Adenocarcinoma	IB
12	M	65	Squamous cell carcinoma	IIB
13	M	54	Large cell carcinoma	IB
14	M	60	Adenocarcinoma	IIIA
15	M	75	Squamous cell carcinoma	IIB

[a]The pathologic stage was classified according to the American Joint Committee on Cancer (AJCC).

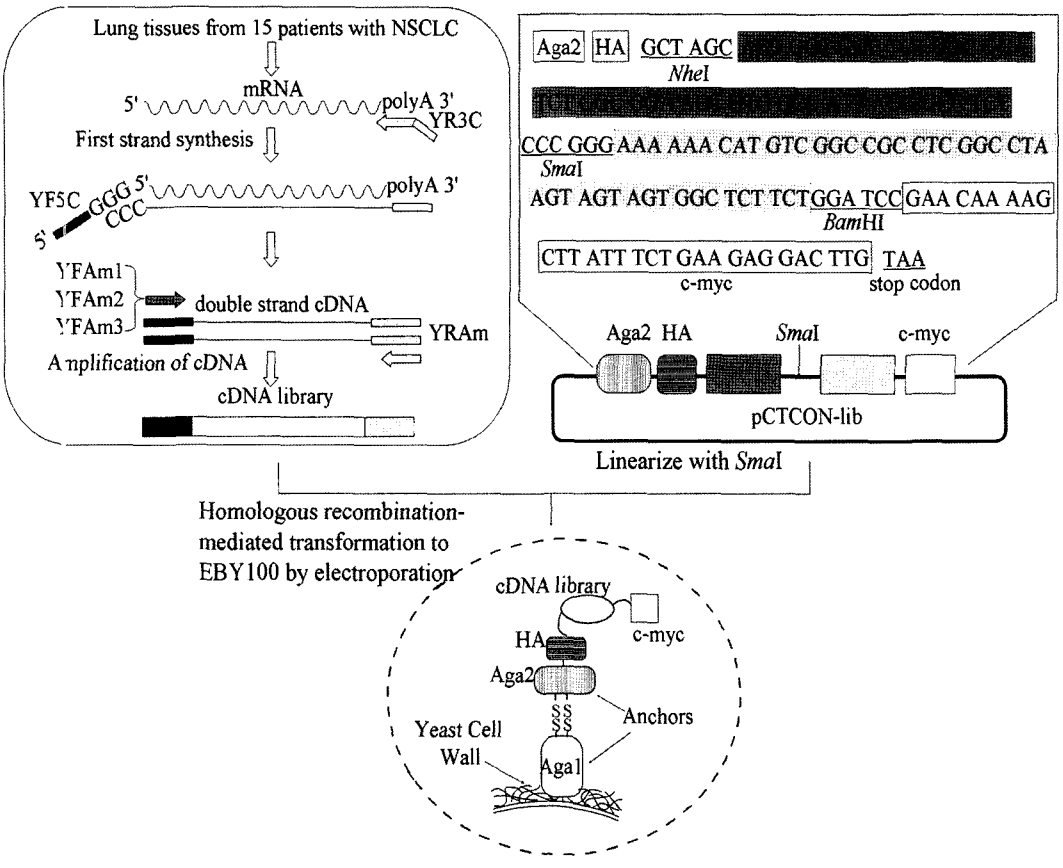

Fig. 1. Schematic diagram showing the overall procedures of construction of the cDNA library from lung cancer tissues on the yeast surface display system.

The identical sequence regions for the homologous recombination between pCTCON-lib and cDNA library are indicated with dark and light gray colors, respectively. The detailed procedures are described in the Materials and Methods section.

used were slightly modified as follows for subcloning the cDNA library into the yeast surface display vector (Fig. 1). The first and double strand cDNAs were synthesized from ~2 μg mRNA using primer sets of YF5C (5'-AGT GGT GGC TCT GGC GGA TCT GGC GGA AGT GTG GCC ATT ACG GCC TCT CCC GGG-3') and YR3C (5'-AGA AGA GCC ACT ACT ACT TAG GCC GAG GCG GCC GAC ATG TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT VNN-3', where V=A, G, or C, and N=A, G, C, or T). For the amplification of cDNA, three forward primers (YFAm1, YFAm2, and YFAm3) and a reverse primer (YRAm) were mixed together to allow translation of each cDNA in all of the three open reading frames with Aga2 on a yeast display vector. The sequence of each primer is as follows: YFAm1 (5'-GGT GGC TCT GGC GGA TCT GGC GGA AGT GTG-3'), YFAm2 (5'-GT GGC TCT GGC GGA TCT GGC GGA AGT GTG-3'), YFAm3 (5'-T GGC TCT GGC GGA TCT GGC GGA AGT GTG-3'), and YRAm (5'-AGA AGA GCC ACT ACT ACT TAG GCC GAG-3'). A total of 5 reactions for the double-strand cDNA synthesis and 49 reactions (5 PCR cycles) for the amplifications of cDNA were carried out. To allow homologous recombination of the amplified cDNA inserts with a yeast surface display vector by gap repair function [3], the parental pCTCON plasmid was modified to have YF5C primer and YR3C primer sequences at the upstream and downstream of cDNA insert site (generating by cutting with SmaI), respectively, resulting in a pCTCON-lib vector (Fig. 1). The amplified cDNAs were concentrated using Pellet Paint (Novagen) [12] and quantified by absorbance at 260 nm. The cDNA insert (10 μg) was mixed with linearized pCTCON-lib (1 μg) by SmaI digestion, and transformed into the yeast EBY100 strain by homologous recombination using a Bio-Rad Gene Pulser electroporation apparatus [3, 12, 13]. A total of 6 transformations were performed in parallel. The transformants were pooled and propagated at 30°C for 20 h directly in liquid selective SD-CAA media (-ura, -trp), which contained 20 g/l glucose, 6.7 g/l yeast nitrogen base without amino acids (Difco, U.S.A.), 5.4 g/l Na_2HPO_4, 8.6 g/l $NaH_2PO_4 \cdot H_2O$, and 5 g/l casamino acids (Difco) [3, 12, 13]. Cell surface expression of cDNA library was induced at 30°C for 20 h in selective media of SG-CAA, which contained the same composition as SD-CAA, except glucose was replaced with galactose. The library size was

determined by plating serial 10-fold dilutions of the transformed cell on the selective SD-CAA agar plates.

Pre-absorption of Patient and Normal Sera

To be used as a screening probe, normal sera from 8 healthy donors and patient sera from 15 patients were pooled together, respectively, before the following pre-absorption treatment. Induced yeast cells (~5×10^7 cell/ml) transformed with the empty vector, pCTCON-lib, were pelleted by centrifugation and washed twice with PBSB (10 mM Na-phosphate, pH 7.4, containing 100 mM NaCl and 1 mg/ml BSA). The cells were resuspended with 500 μl diluted (1:50) normal or patient sera and then incubated at 4°C for 30 h with gentle agitation to remove nonspecific-binding antibodies to yeast cell surface components [15, 22]. The supernatant was recovered by centrifugation. After adding sodium azide [0.01% (w/v)] for preservation, the pre-absorbed sera were stored at −70°C until used.

Magnetic Activated Cell Sorting (MACS)

A Miltenyi MidiMacs system with LS columns with anti-biotin or streptavidin microbeads was used for magnetic activated cell sorting (MACS) following the protocol described by Siegel *et al.* [18]. Induced yeast cells (~10^9 cells) expressing the cDNA library were incubated with pre-absorbed normal (for depletion sorting) or patient (for positive sorting) sera, both of which were diluted 500-fold, at 25°C for 30 min in 1 ml of PBSB. Cells were washed with ice-cold MACS buffer (PBSB+2 mM EDTA) and incubated with biotin-conjugated goat anti-human IgG (1:150 dilution) (Sigma) at 25°C for 30 min in 1 ml of MACS buffer. After washing twice with 1 ml of MACS buffer, the cells were resuspended with 1 ml of MACS buffer, mixed with anti-biotin microbeads (100 ml), and then incubated with rotations at 10°C for 10 min [18]. After adding 6 ml of MACS buffer, a suspension of cells (~7 ml) was loaded onto a Miltenyi Macs LS column at 25°C, held in place in the external magnetic field. By washing three times with 3 ml of MACS buffer, the nonbound fraction was collected as nonbinding cells. After removal of the column from the magnetic field, the bound cells were eluted with 9 ml of MACS buffer as binding cells. Collected cells from the nonbound fraction (for depletion sorting) or bound fraction (for positive sorting) were regrown and induced at 30°C as above, and then sorted repeatedly with the same procedures.

Flow Cytometric Analysis and Sorting (FACS)

Yeast cell labeling for affinity analysis and sorting by flow cytometry was performed as described before [3, 12, 13]. Induced yeast cells (~10^7 cells) were incubated with diluted normal or patient sera (exact dilution fold is specified in the figure legends) at 25°C for 30 min in 0.2 ml of PBSB with frequent gentle mixings. After washing with 0.5 ml of ice-cold PBSB, cells were labeled with FITC-conjugated goat anti-human IgG (1:50 dilution) in 0.1 ml of PBSB for 20 min on ice with frequent mixings. The labeled cells were washed, resuspended with PBSB, and then analyzed or sorted on a BD FACS Calibur (Becton Dickinson). Typically, the top 0.05–0.1% of sero-positive cells was sorted. The sorted cells were regrown and induced at 30°C, and sorted repeatedly with the same labeling procedures. The finally sorted yeast cells were plated on the selective SD-CAA agar plates and allowed to grow for 48 h at 30°C. Individual clones were isolated and characterized further. For monitoring the yeast-surface expression level of the cDNA library, the same labeling procedures were performed with mouse anti-c-myc 9e10 mAb (1:100 dilution) and R-phycoerythrin-conjugated goat anti-mouse IgG (1:25 dilution).

Sequence Analysis of Individual Clone

Surface display plasmids from selected yeast cells were recovered by using the Zymoprep yeast plasmid miniprep kit (Zymo Research) as described previously [3]. Rescued plasmids were transformed into *E. coli* XL1-Blue competent cells by electroporation, amplified, and purified using a Miniprep kit (Intron Biotech., Seoul, Korea). cDNA inserts in pCTCON-lib were sequenced using a forward primer (5'-GTT CCA GAC TAC GCT CTG CAG G-3') and a reverse primer (5'-GAT TTT GTT ACA TCT ACA CTG TTG-3') at the Bionics Co. (Seoul, Korea). Protein identity and amino acid sequence motif searches were performed using the BLASTP tool in the NCBI Web site (http://www.ncbi.nlm.nih.gov/BLAST/). The nucleotide sequence was also blasted in the Cancer Immunome Database (http://www2.licr.org /CancerImmunomeDB/) [20].

RESULTS AND DISCUSSION

Construction of cDNA Library on the Yeast Cell Surface

Isolated mRNAs from lung tissues derived from 15 NSCLC patients with different histologic types and pathologic stages were pooled together for cDNA library construction with an attempt to efficiently isolate diverse antigens associated with the complicated NSCLC (Table 1). The cDNA library was constructed to be expressed in all of the three open reading frames on the yeast-cell surface (Fig. 1). When the library size was determined by serial 10-fold dilutions of the transformed yeasts on the selective SD-CAA plates, it was approximately 5×10^6. When individual plasmids were rescued from randomly chosen 24 clones in the unselected library, all of the cDNA inserts exhibited authentic sequences (data not shown), indicative of fidelity of the library diversity. The cDNA insert size ranged from 200 bp to 1.5 kb on 1% agarose gels (Fig. 2A), with a high frequency toward sizes of ~300–800 bp, although the sizes of the amplified cDNA library prior to transformation were evenly distributed from 200 bps to 6 kb (data not shown).

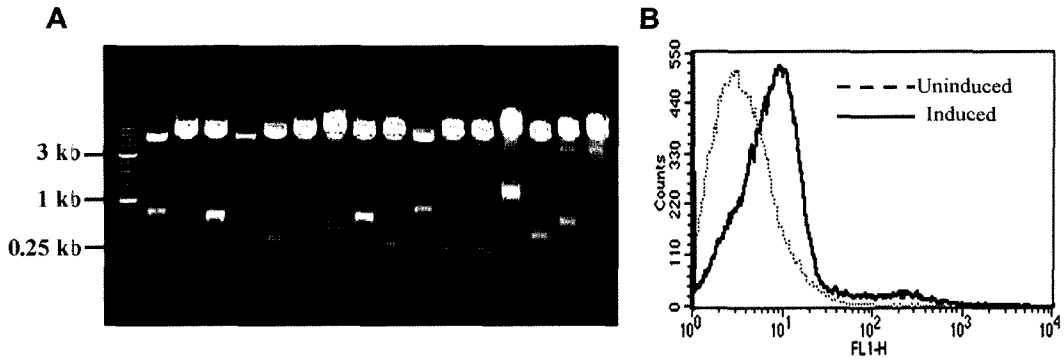

Fig. 2. A. Representative agarose gel electrophoresis to determine the insert size of a cDNA library constructed on the yeast cell surface. Individual plasmids isolated from randomly chosen clones from the unselected library were digested with NheI and BamHI and then analyzed on 1% agarose gel. **B.** Histodiagram showing the expression level of the cDNA library on the yeast surface monitored by flow cytometry. The expression of cDNA on the yeast surface was monitored by single labeling with anti-c-myc mouse mAb/FITC-labeled anti-mouse IgG (*x*-axis) on the uninduced or induced yeast-cells-transformed cDNA library.

The cell surface expression of cDNA library was monitored by indirect immunofluorescent labeling using mouse 9e10 mAb specifically recognizing the C-terminal c-myc tag and subsequent flow cytometric analysis [3, 13]. Compared with uninduced control cells, induced cells at 30°C for 20 h exhibited significant positive labeling for a C-terminal c-myc epitope tag, indicating that the cDNA library was expressed well on the yeast surface (Fig. 2B). Varying the induction conditions, such as temperature of 20, 25, 30, and 37°C and induction duration of 10, 20, and 30 h, did not make any significant differences in the expression profiles of the cDNA library (data not shown).

Effects of Pre-absorption and Dilution Fold of Patient Sera on Serum Reactivity with cDNA Library

Sera from 15 patients with NSCLC were pooled together. Before being used as a screening probe, the pooled patient sera were first treated with a pre-absorption procedure using induced yeast cells transformed with the empty vector, pCTCON-lib, to remove antibodies reactive with yeast-cell surface components [22, 23]. The total abundance of IgG in a human is about 15 mg/ml, accounting for ~80% of the total serum immunoglobulins and ~20% of the total serum proteins [4]. Thus, to find the optimal serum dilution fold during the screening procedure, the pooled patient sera that were untreated or pre-absorbed were serially titrated to compare the reactivity with uninduced and induced yeast cells expressing the cDNA library. Untreated patient sera exhibited significant reactivity with both uninduced and induced yeast cells, exhibiting ~1.3-fold higher reactivity with induced cells in the dilution range of up to 2,000-fold, judged from the mean fluorescence intensity (MFI) (Fig. 3). However, preabsorbed patient sera showed ~1.6–2.6-fold higher reactivity with induced cells in the dilution range of 50–2,000-fold, compared with uninduced cells that showed a negligible, background level of reactivity (Fig. 3). Thus, preabsorption treatment of patient sera was required to remove antibodies that are reactive with yeast-cell surface components. The optimal sera dilution of up to 2,000-fold was also identified for the next screening procedure.

Positive and Depletion Sortings by MACS and FACS

To isolate patient sera-specific antigens from the cDNA library expressed on the yeast surface, positive selections with combinations of MACS and FACS were carried out. Initial three rounds of MACS using the pre-absorbed patient sera (500-fold diluted) were performed to rapidly

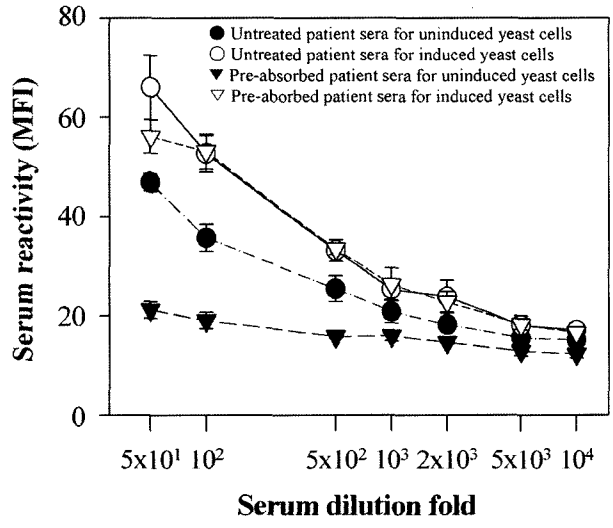

Fig. 3. Reactivity of patient sera for uninduced or induced yeast cells expressing the cDNA library.
The reactivity of patient sera that were pooled from 15 patients and then diluted by the indicated fold before (*untreated*) and after the pre-absorption treatment (*pre-absorbed*) was indicated as mean fluorescence intensity (MFI) by flow cytometric analysis after single labeling with patient sera/FITC-conjugated goat anti-human IgG on the uninduced or induced yeast cells. Error bars indicate the standard error for triplicate experiments.

enrich cells expressing patient sera-positive antigens, which were further enriched by three rounds of FACS [13, 18]. Weak cross-reactivity of patient sera with R-phycoerythrin-conjugated goat anti-mouse IgG was observed (data not shown), limiting the usage of anti-c-myc mouse 9e10 mAb. Furthermore, inserted clones might not express c-myc epitope tag because of frame shifts or stop codons introduced during cDNA library construction. Thus, throughout the positive selections by FACS, cells were singled labeled with the pre-absorbed patient sera (1,000-fold diluted) and secondary FITC-conjugated anti-human IgG and sorted by setting a sorting gate to select the top ~0.05–0.1% cells. As shown in Fig. 4A, cells expressing patient sera-positive antigens were gradually enriched, as indirectly shown by increases of MFI obtained by setting a same gate. To determine whether enriched cells from each round of MACS and FACS were patient sera-specific, their reactivity with pooled normal sera from 8 healthy donors that were pre-absorbed was assessed by flow cytometry. Serum reactivity of the selected cells was compared by the relative ratio of MFI for patient sera to normal sera. In contrast to previously reported results [22], the selected cells from each round of MACS and FACS did not show any significant differences in serum reactivity between patient and normal sera (Figs. 4A and 4C). Serum dilutions from 50- to 2,000-fold also showed the indistinguishable serum reactivity between patient and normal sera (data not shown).

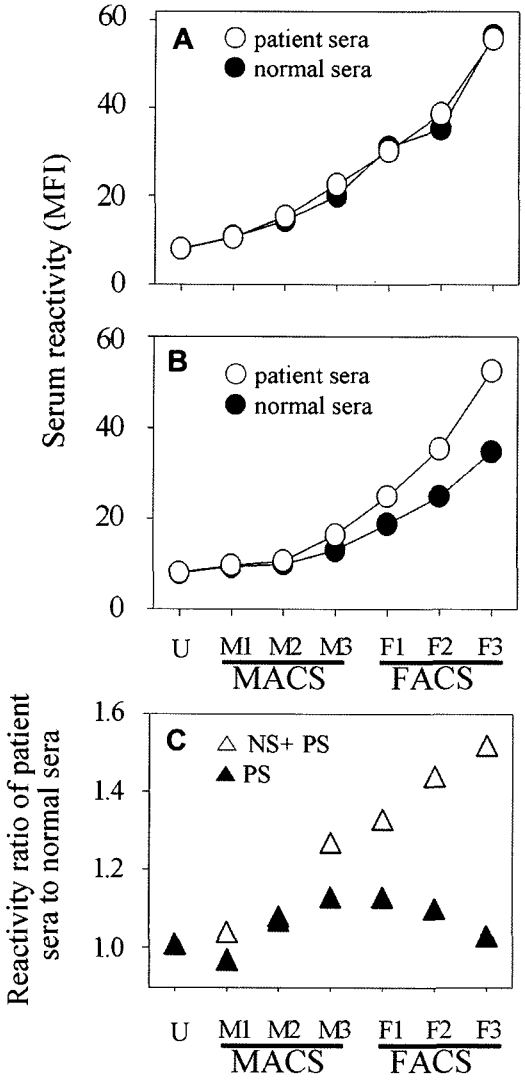

Fig. 4. Comparisons between only positive selection (*PS*) with patient sera (**A**) and the combined selections of negative selection (*NS*) with normal sera and positive selection (*PS*) with patient sera (**B**) for the enrichment efficiency of patient sera-specific antigens (**C**).
The serum reactivity was indicated as mean fluorescence intensity (MFI) by flow cytometric analysis after labeling with pooled, pre-absorbed normal and patient sera (500-fold diluted) on the unselected cells (indicated as *U*) and cells after each round of MACS (*M1*, *M2*, and *M3*) and FACS (*F1*, *F2*, and *F3*). In (**C**), the reactivity ratio of patient sera to normal sera was estimated by the relative ratio of MFI for patient sera to normal sera obtained from (**A**) and (**B**).

To address the nonselectivity of isolated antigens from the positive selections for both patient and normal sera, we introduced depletion sorting procedures, where pre-absorbed normal sera (500-fold diluted) were probed during MACS to remove antigens reactive with normal sera, and subsequently carried out positive selections by FACS to enrich patient sera-specific antigens using patient sera (1,000-fold diluted) as a screening probe (Fig. 5). Three successive depletion sortings by MACS with normal sera gradually enriched cells selectively reactive with patient sera (Fig. 4B and Fig. 5), populating cells with a ~1.3-fold higher reactivity ratio of patient sera to normal sera after the third round of MACS (Fig. 4C). Subsequent positive selections by FACS using patient sera further enriched cells preferentially reactive with patient sera (Fig. 5), isolating cells exhibiting a ~1.5-fold higher reactivity for patient sera over normal sera after the final round of FACS (Fig. 4C). These results demonstrated that the initial three rounds of negative selection with normal sera effectively removed antigens reactive with normal sera, and the subsequent positive selections with patient sera effectively enriched patient sera-selective antigens.

Serological Analysis of Selected Clones

Individual yeast clones were isolated by plating the selected cells from the final round of sorting, and the individual plasmids were rescued. DNA sequencing of 110 isolated clones identified 8 unique clones, designated sequentially as LCB1 to LCB8 antigens. The isolated plasmids were individually retransformed into yeast EBY100 cells to ensure antigen monoclonality. The individual antigens expressed on the yeast cell surface were further assessed for the specific reactivity with pooled patient and normal sera. All antigens, particularly LCB1 to LCB6, exhibited preferential reactivity with patient sera to normal sera (Fig. 6A). Furthermore, instead of pooled sera, individual

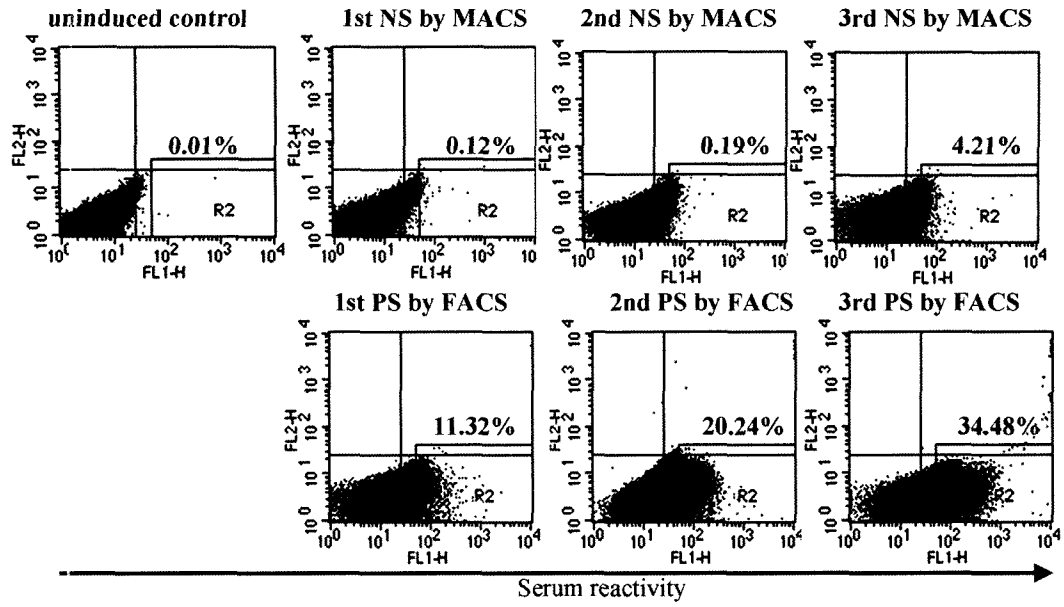

Fig. 5. Enrichment profiles of patient sera-positive antigens during negative selection (*NS*) by MACS with pre-aborbed normal sera and subsequent positive selection (*PS*) by FACS with pre-absorbed patient sera.
After each round of MACS and FACS, yeast cells were analyzed by flow cytometry after single-labeling with pre-absorbed sera (500-fold diluted)/FITC-conjugated goat anti-human IgG (*x*-axis). The indicated percentage was the frequency of positive population entering the R2 gate. Data for the uninduced control yeast cells are shown as a negative control.

sera from 10 patients with NSCLC were compared with individual normal sera from 5 healthy donors for reactivity with the isolated antigens. As shown in Fig. 6B, individual patient sera exhibited a more than 2-fold higher reactivity with the most antigens compared with normal sera, the reactivity of which was only marginally above or around the background level. Taken together, the results showed that all of the isolated antigens showed patient sera-specific reactivity.

Sequence Analysis of Selected Clones

The fusion of the cDNA library with the cell-surface-anchoring motif of the Aga-2 protein directly identified the open reading frame of the inserted antigen. The selected 8 antigens encoded proteins with 18–143 amino acid residues. BLASTP analyses with the amino acid sequences led to the identification of 3 antigens (LCB1, LCB2, and LCB5) with known proteins and 3 antigens (LCB3, LCB4, and LCB6) with unknown proteins showing sequence identity of 80–95% in the GenBank and EST databases (Table 2). LCB7 and LCB8 exhibited no sequence identity of more than 70% to current GenBank entries. Somatic mutations are a hallmark of human cancer. Thus, it is not surprising that isolated cDNA fragments did not show 100% identity with proteins in the databases. For example, a recent study showed that the average frequency of random mutations in tumors was 2.2×10^{-6} per bp and thus the entire tumor could harbor as many as 10^{12} different single-nucleotide substitutions [1].

LCB1 represents DOCK4 protein, a GTPase activator, which enhances the formation of intercellular junctions [24]. The mutations of DOCK4 protein have been identified in a subset of human cancer cells, such as ovarian, prostate, glioma, and colorectal cancer cells [24]. Thus, LCB1 might be associated with tumor metastasis in NSCLC. LCB2 and LCB5 correspond to the Hepatitis B virus receptor-binding protein and FLJ00385, respectively, both of which belong to the immunoglobulin heavy constant region subfamily. Though lower levels of surface expression of the immunoglobulin heavy constant region subfamily have been closely linked with chronic lymphocytic leukemia [21], their implication with NSCLC should be elucidated further.

To determine further whether the isolated antigens were already deposited by previous SEREX, the nucleotide sequence was also analyzed in the Cancer Immunome Database [20], which contains more than 2,000 tumor antigens isolated by SEREX from cDNA expression libraries derived from various cancer cells and/or tissues. Five antigens showed high sequence homology of more than 95% identity with the reported cDNA clones (Table 2). In particular, LCB3, LCB4, LCB7, and LCB8 corresponded to cDNA clones of Hom-Br4-91, TL15-37, Hom-Br4-91, and NY-TLU-40, respectively, which were all associated with lung cancer (Table 2). Hom-Br4-91 represents actinin-4, which is an actin-bundling protein involved in cell motility [8]. The inactivation of acinin-4 abolished the metastatic potential of human cancers, but its cytoplasmic expression enhanced cell motility in several cancer cells, suggesting that actinin-4 is implicated in cancer invasion [8]. TL15-37 represents sorting nexin 13 protein (SNX13), which is involved in

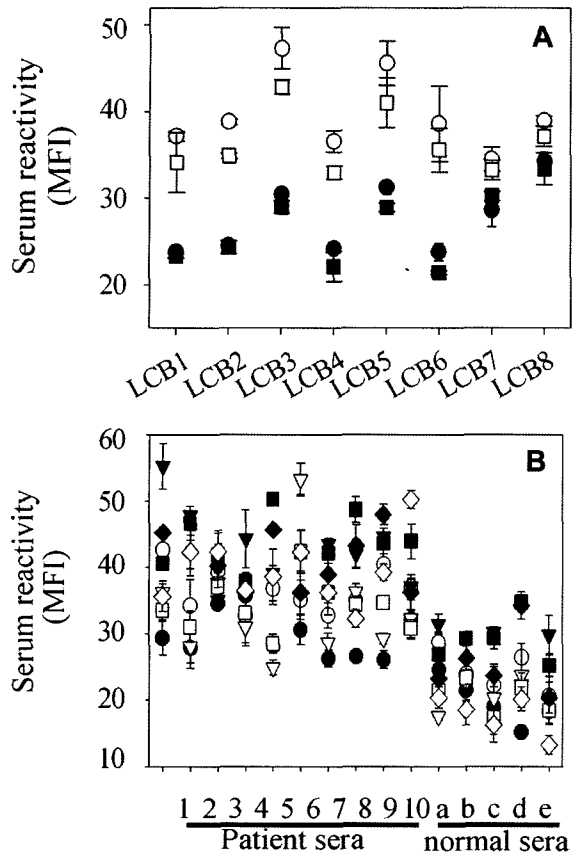

Fig. 6. Serological analysis of isolated clones.
A. Eight isolated clones (LCB1-LCB8) were analyzed for the serum reactivity with pooled, pre-absorbed normal (●, ■) and patient (○, □) sera diluted by 500-fold (●, ○) or 2,500-fold (■, □). **B**. The isolated eight clones, LCB1 (●), LCB2 (○), LCB3 (▼), LCB4 (▽), LCB5 (■), LCB6 (□), LCB7 (◆), and LCB8 (◇), were analyzed for the serum reactivity with pre-absorbed, 10 individual patient sera (*1-10*) and 5 individual normal sera (*a-e*), which were diluted 500-fold. The serum reactivity was indicated as mean fluorescence intensity (MFI) by flow cytometric analysis. Error bars indicate the standard error for triplicate experiments. The differences in serum reactivity of the isolated 8 clones between patient sera (n=10) and normal sera (n=5) were statistically significant at the P<0.0003 level.

intracellular trafficking of receptor tyrosine kinases [25]. For instance, stable expression of SNX13 delayed lysosomal degradation of the epidermal growth factor receptor [25]. NY-TLU-40 is related with CDO, a cell surface receptor of the immunoglobulin/fibronectin type III repeat family involved in myogenic differentiation [10]. Taken together, even though direct linkages between the isolated clones and NSCLC should be validated further, the newly isolated antigens might be implicated in NSCLC because they are all involved in cellular signaling related with cell motility, growth, and differentiation.

As we described here, SEREX with patient sera alone would isolate normal cellular antigens that are recognized by autoantibodies in both patient and normal sera. These antigens will not be suitable as therapeutic targets for either vaccine or antibody based approaches because of possible autoimmune diseases [9]. In this study, we demonstrated an optimal screening strategy for the isolation of patient sera-specific antigens from a yeast surface-displayed cDNA library derived from lung cancer tissues, using autologous patient and allogeneic normal sera. We isolated 3 known and 5 unknown antigens, which are potentially associated with NSCLC. Isolation of patient sera-specific antigens that were not identified yet by the conventional SEREX, but possibly associated with lung cancer, validates our combined selection methodology of negative selections using allogeneic normal sera with positive selections with autologous patient sera. Although the newly isolated antigens should be further validated for the expression profiles in lung cancer and normal tissues, they might be potential candidates for diagnostic markers and therapeutic targets in NSCLC.

Acknowledgments

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Table 2. List of cDNA clones isolated from the yeast-surface displayed cDNA library derived from lung tissues of 15 patients with NSCLC.

Clones	BLASTP		CIDB	
	GeneBank Accession No.	Description	SEREX clone No.	Associated tumor type
LCB1	AAI17695	DOCK4 protein	Mz19-16a	Skin
LCB2	AAS88328	Hepatitis B virus receptor-binding protein	NM	
LCB3	BAC05016	Unknown protein	Hom-Br4-91	Lung
LCB4	AAL55794.1	Unknown protein	TL15-37	Lung
LCB5	BAC03445	FLJ00385 protein	NM	
LCB6	GI:33317107	Unknown protein	NM	
LCB7	NM[a]		Hom-Br4-91	Lung
LCB8	NM		NY-TLU-40	Lung

[a]NM: Clones showing below 70% sequence identity with proteins (BLASTP) or nucleotide sequences (CIDB) in the database were designated as not matched (NM).

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