Isolation and characterization of human lung cancer antigens by serological screening with autologous antibodies.

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ABSTRACT

Serological analysis of a recombinant cDNA expression library (SEREX) derived from two lung adenocarcinoma cancer cell lines using autologous sera led to the isolation of 41 positive cDNA clones comprising 28 different antigens. They coded for a variety of nuclear and cytoplasmic proteins. Among the antigens, nucleoporin 107 (NUP107) was isolated most frequently (5 of 41 clones). The second most frequently isolated antigen was coded for by C21orf58 (4 of 41 clones). During serological analysis of selected antigens based on their reactivity to sera from normal individuals and lung cancer patients, none of the antigens showed a cancer-restricted recognition pattern. However, 5 genes including *NUP107* showed higher expression when we examined the changes in gene expression in 5 different adenocarcinoma cell lines, including those used in SEREX, compared with their levels in normal lung tissues by cDNA microarray analysis. On the other hand, the expression levels of 5 genes including *C21orf58* were down regulated in all adenocarcinoma cell lines. This SEREX study combining comprehensive gene expression assays has added to the growing list of lung cancer antigens, which may aid the development of diagnostic and immunotherapeutic reagents for patients with lung cancer.

1. Introduction

Lung cancer is one of the leading causes of cancer death, and its 5-year survival rate is poor, even in the early stages [1, 2]. Recent progress in tumor immunology based on the molecular identification of tumor antigens has suggested that immunotherapy has potential as a promising treatment for lung cancer.

Serological analysis of recombinant cDNA expression libraries (SEREX) was developed to combine serological analysis with antigen cloning techniques in order to identify human tumor antigens that eliciting high-titer IgG antibodies [3, 4]. SEREX has contributed greatly to our understanding of the humoral immune response to cancer [5]. It has led to the identification of a variety of tumor antigens, including cancer/testis (CT) antigens [6, 7], mutational antigens [8], over-expressed antigens [9, 10], differentiation antigens [6, 11], splice-variant antigens [12], and viral antigens [13].

Using the SEREX approach in lung cancer, several previously unknown lung cancer antigens have been identified and characterized [14-17]. In our previous studies, we isolated a CT antigen, XAGE-1, in lung adenocarcinoma by SEREX analysis. In the XAGE-1 transcripts, XAGE-1b was the dominant antigen and was found to be highly immunogenic for antibody production in lung adenocarcinoma patients [18, 19]. In the present study, we applied the SEREX methodology to 2 lung adenocarcinoma cell lines in combination with comprehensive gene expression assays in order to add to the repertoire of lung cancer antigens.

2. Materials and Methods

2.1. Tissues, cell lines, and sera

Tumor specimens were surgically obtained from patients at Kawasaki Medical University Hospital. Lung cancer and adjacent normal lung tissues were obtained in the same samples from 5 specimens. OU-LU-6, -11, -14, -17, and -26 were lung cancer cell lines established from the pleural effusions of male patients with lung adenocarcinoma. Sera were obtained from 32 lung cancer patients and 27 healthy donors. Written informed consent was obtained from all patients and healthy donors involved in our study in accordance with the university guidelines.

2.2. Preparation of the cDNA library from the lung cancer cell lines

mRNA was purified from the two lung adenocarcinoma cell lines OU-LU-11 and OU-LU-17 using a Quick Prep mRNA Purification Kit (Amersham Pharmacia, Piscataway, NJ). Then, a cDNA expression library was prepared in a γZAP Express vector using a cDNA library kit (Stratagene, La Jolla, CA).

2.3. Immunoscreening of cDNA libraries

cDNA expression libraries of lung cancer cell lines were screened with autologous patient sera. The screening procedure was described previously [18, 20]. In brief, serum samples that had been diluted 1:10 were preabsorbed with lysate from *Escherichia coli*

Y1090/Y1089 and coupled to Sepharose 4B (BioDynamics Lab Inc., Tokyo, Japan). Recombinant phages (approximately 4,000 pfu) on agar in a plastic dish (140 mm diameter) were amplified for 8 hr and then transferred to 135 mm diameter nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany) for 15 hr at 37°C. The membranes were then blocked with 5% non-fat milk and pre-screened by incubation with peroxidase-conjugated Fc fragment-specific goat anti-human IgG (Jackson ImmunoResearch, West Grove, PA, USA) (1:2,000 dilution) for 1 hr at room temperature. Color was developed using 3, 3' diaminobenzidine (Sigma, St. Louis, MO, USA), and IgG-encoding clones were marked so that they could be excluded from subsequent examinations. The membranes were then incubated overnight at room temperature with the preabsorbed serum diluted to 1:200. The membranes were incubated with peroxidase-conjugated Fc fragment-specific goat anti-human IgG (Jackson ImmunoResearch) (1:2,000 dilution) for 1 hr at room temperature and color was developed. Positive clones were collected and subcloned to monoclonality by 2nd and 3rd screenings using 82 and 47 mm diameter membranes, respectively. A randomly chosen negative clone was included in each assay as a negative control. Phage plaque assays were performed to test the reactivity of sera (1:200 dilution) from healthy donors and lung cancer patients against SEREX-defined clones. Exponentially growing *E. coli* XL-1 Blue MRF' were infected with 20-30 pfu of cloned phages encoding individual SEREX-defined antigens and then mixed with 0.7% NZY agarose/2.5mM IPTG.

Bacterial suspensions (100 μl) containing cloned phages encoding SEREX-antigens were then plated on 1.5% NZY agar in plastic dishes (140 mm diameter), amplified for 8 hr, and transferred to nitrocellulose membranes for 15 hr at 37°C. Color was developed as described above.

2.4. Sequence analysis of reacted clones

The clones that reacted positively were subcloned to monoclonality, purified, and excised *in vivo* to pBK-CMV plasmid forms (Stratagene). The plasmid DNA was prepared using the Quantum Prep Plasmid Miniprep Kit (Bio-Rad, Hercules, CA). The nucleotide sequences of the cDNA inserts were determined using ABI 3130x1 Genetic Analyzer (Applied Biosystems Foster City, CA), and sequence alignment was performed with BLAST software and sequences in the GenBank database.

2.5. Total RNA isolation and cDNA synthesis

Total RNA was isolated from the tumor tissues using an RNeasy Mini Kit (Qiagen, Hilden, Germany). Total RNA from normal tissues was obtained commercially (BD Bioscience Clontech, Palo Alto, CA, USA). The RNA (2 μg) was reverse-transcribed into single-strand cDNA using Moloney murine leukemia virus reverse transcriptase (Ready-To-Go You-Prime First-Strand Beads, GE Healthcare, Buckinghamshire, UK) and oligo$(DT)_{15}$ as a primer. The cDNA samples were tested for integrity by amplification of *G3PDH* in a 30-cycle reaction.

2.6. Gene expression microarrays

Gene expression was examined using Agilent Human 1A oligomicroarrays containing 60-mer DNA probes in a 22K format (Agilent Technologies, Palo Alto, CA). Of 19,061 spots, 18,086 are non-controls, and there are 17,086 unique transcript sequences from 15,989 unique human genes. Five hundred nanogram of total RNA from lung adenocarcinoma cell lines for the test samples and normal lung tissue for the reference samples were used to synthesize labeled cRNA (Low RNA Input Linear Amp Kit, Agilent Technologies) in the presence of cyanine 3-dCTP and cyanine 5-dCTP (Perkin-Elmer Life Sciences, Boston, MA), respectively. Differentially labeled test and reference samples were mixed with Agilent control targets before being hybridized onto the oligomicroarrays for 17 hr at 60°C in a rotating oven. The fluorescence intensities of the targets were detected using a laser confocal scanner (Agilent Technologies), and the resulting images were processed using the Feature Extraction Software, version 9.1 (Agilent Technologies).

2.7. Quantitative real-time RT-PCR

Two-step real-time RT-PCR was performed using a StepOne Real-Time PCR System (Applied Biosystems). cDNA was synthesized using a High-capacity cDNA Reverse Transcription kit (Applied Biosystems). TaqMan Gene Expression Assays (Applied Biosystems) were used to measure the mRNA levels of *MYL6B* (Assay ID: Hs00365997_g1), *MET* (assay ID: Hs01565580_m1), *NUP107* (Assay ID:

Hs00914852_m1), *HSPA4* (Assay ID: Hs00382884_m1), and *SCOC* (Assay ID: Hs00261486_m1). mRNA levels were expressed as n-fold differences relative to *G3PDH* (internal standard) and their levels in normal testis tissues (calibrator). PCR was performed using TaqMan PCR Master Mix (Applied Biosystems), and the thermal cycling conditions involved initial denaturation at 95°C for 10 min followed by 50 cycles of 95°C for 15 sec and 60°C for 1 min. The parameter Ct was defined as the threshold cycle number in which the fluorescence generated by the cleavage of the probe passed above the baseline. The target mRNA was quantified by measuring its Ct value, and transcripts of *G3PDH* were quantified as an endogenous RNA control using TaqMan human *G3PDH* control reagents (Applied Biosystems).

3. Results

3.1. Autologous serum screening of cDNA library

A cDNA expression library of 1.2 x 10^6 clones was prepared from the OU-LU-11 lung adenocarcinoma cell line. Immunoscreening 1.6 x 10^5 clones with autologous patient serum yielded a total of 21 positive clones. The nucleotide sequences of the cDNA inserts identified 19 different genes, which were designated as OY-LC-17 to OY-LC-35 (Table 1). OY-LC-19 was found in 3 overlapping clones. OY-LC-25 and OY-LC-28 were found in 2 overlapping clones. The others were all isolated from single clones.

A cDNA expression library of 3 x 10^6 clones was prepared from the OU-LU-17 lung adenocarcinoma cell line. Immunoscreening 2 x 10^5 clones with autologous patient serum yielded a total of 20 positive clones. The nucleotide sequences of the cDNA inserts

identified 9 different genes, which were designated as OY-LC-36 to OY-LC-44 (Table 2). OY-LC-36, nucleoporin 107 (NUP107), was the most frequently isolated antigens, being found in 5 overlapping clones. OY-LC-37, chromosome 21 open reading frame 58 (C21orf58), was the second most frequently isolated, being found in 4 overlapping clones.

All genes showed universal expression in adult normal tissues in the UniGene database.

3.2. Reactivity of allogeneic sera with SEREX-defined antigens

The reactivity of sera from healthy donors and lung cancer patients with the randomly selected 17 SEREX-defined antigens was investigated using a phage plaque assay (Fig. 1). Allogeneic serum samples were obtained from 27 healthy donors and 32 lung cancer patients. As shown in Table 3, all of the 17 antigens screened reacted with a subset of sera from both healthy donors and lung cancer patients.

3.3. Gene expression profiles

Gene expression analysis was performed on 5 lung adenocarcinoma cell lines including those used in SEREX, using cDNA microarray. Of 28 SEREX-defined genes, 5 genes, *MYL6B*, *MET*, *NUP107*, *HSPA4,* and *SCOC,* showed higher expression levels in all 5 different cell lines compared with normal lung tissues. *MYL6B* expression was up regulated more than 2-fold in all cell lines (Table 1 and 2). On the other hand, the expression of 5 genes, *WWC2*, *TJP2*, *SAMSN1*, *C21orf58,* and *TPT1,* was down regulated

in all cell lines.

3.4. mRNA expression in normal tissues and tumors

To investigate the expression of *MYL6B*, *MET*, *NUP107*, *HSPA4,* and *SCOC* mRNA, we performed quantitative real-time RT-PCR analysis using the gene specific TaqMan probes. As shown in Fig. 2, lower levels of the *MYL6B*, *NUP107,* and *HSPA4* transcripts were observed in normal, nongametogenetic tissues than in the normal testis tissues. On the other hand, considerably higher mRNA expression levels of *MET* and *SCOC* were observed in some normal tissues compared to those in the normal testis tissue. *MYL6B*, *NUP107,* and *HSPA4* mRNA expression was further examined in 5 lung cancers and the adjacent normal tissue by quantitative real-time RT-PCR. However, no distinct differences in their expression levels were observed between the cancers and adjacent normal tissue (Fig. 3).

4. Discussion

The 28 antigens identified by our SEREX analysis of 2 lung adenocarcinoma cell lines represented a diverse set of proteins. Twenty-five of these antigens were products of known genes, and 3 represented novel gene products.

Of the SEREX-defined genes, 5, *MYL6B*, *MET*, *NUP107*, *HSPA4,* and *SCOC,* showed higher expression when gene expression changes were examined in 5 different adenocarcinoma cell lines, including those used in SEREX, and compared with those in normal lung tissues by cDNA microarray analysis. Using quantitative real-time RT-PCR

analysis, we found that the expression levels of *MYL6B*, *NUP107,* and *HSPA4* mRNA were reduced in normal tissues compared to those in normal testis tissue. Although no significant differences in their expression were detected between the lung cancer tissue and the adjacent normal tissue in the 5 patients examined, one patient showed higher expression levels of all 3 genes in their cancer tissue than those in adjacent normal tissue. In 5 genes, *MYL6B* and *SCOC* were not reported at SEREX database (Table 1 and 2). *MYL6B* encodes a myosin alkali light chain expressed in both skeletal muscle and in non-muscle tissue. *MYL6B* revealed considerably lower expression levels in adult normal tissues compared to normal testis, and showed higher expression in 2 of 5 lung cancers examined. One of positive lung cancer was adenocarcinoma and the other was squamous cell carcinoma.

Serological analysis for antigens was performed on the basis of their reactivity with sera from healthy donors and lung cancer patients. All examined antigens were reacted with sera from a subset of healthy donors and lung cancer patients by phage plaque assay. No antigens showed a cancer-restricted recognition pattern. Although, serum reactivity was not analyzed against *MYL6B* by phage plaque assay. The immunogenicity *MYL6B* will be tested for IgG antibody in large number of lung cancer patients by ELISA using recombinant protein. Autoantibodies to known normal tissue autoantigens or antibodies elicited by antigens related to necrotic tumor products are often found in serum. Although, a significant proportion of the SEREX antigens are autoantigens of this kind, mutated products might also be present in tumor cells, which can elicit antibodies that cross-react with their corresponding non-mutated counterparts in normal cells [8].

In this study, *NUP107* was the most frequently isolated antigen (5 of 41 clones). Nup107 is a member of the nucleoporin family. It is located on the nuclear rim and is an essential component of the nuclear pore complex. The conserved Nup107-160 complex plays a crucial role in organizing nuclear pore complex assembly in the post-mitotic event and in the interphase nuclei [21-23]. Some nuclear pore complex proteins have demonstrated a relationship with tumor aggressiveness and tumorigenesis. *Nup88* overexpression is closely associated with increased aggressiveness of colorectal cancer [24], malignant melanoma [25], and breast cancer [26]. The *Nup98* gene is fused to at least 15 different partner genes by chromosomal translocation in a wide spectrum of hematologic malignancies, including myeloid leukemia, myelogenous leukemia, precursor-T lymphoblastic lymphoma/leukemia and myelodysplastic syndromes [27-31].

Previously, we identified a CT antigen, XAGE-1, in lung adenocarcinoma by SEREX analysis [18]. In the XAGE-1 transcript, XAGE-1b was the dominant antibody production in lung adenocarcinoma patients. Around 10% of lung adenocarcinoma patients showed antibody responses [19]. In antibody positive patients, strong CD4 T cell responses against XAGE-1 were also elicited [32]. CT antigens have become promising target for diagnosis and immunotherapy for patients with various tumors. However, the expression frequency of tumor antigens, including CT antigens is in a range between 5% and 40% depending on the cancer type, and expression is heterogeneous with frequent antigen loss. The development of polyvalent diagnostic marker and cancer vaccine containing epitopes derived from different tumor antigens could overcome the disadvantage of these tumor antigen characteristics. Taken together, this SEREX study

identified new antigens and added to the growing list of lung cancer antigens, which may aid the development of diagnostic and immunotherapeutic reagents for patients with lung cancer.

Conflict of Interest Statement

All authors disclosed none financial and personal relationships with other people or organizations that could inappropriately influence their work.

Acknowledgments

The authors thank Mr. H. Okamoto, Mr. T. Iwasa, and Mr. Y. Isomoto (Central Research Laboratory, Okayama University Medical School) for performing the DNA sequencing and cDNA microarray analysis. This work was supported in part by a Grant-in Aid for Scientific Research (C) from the Japan Society for the Promotion of Science.

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Figure legends

Figure 1. Phage plaque assay. Seventeen different bacterial suspensions (100 μl) each containing cloned phages (1, OY-LC-17; 2, -19; 3, -23; 4, -25; 5, -28; 6, -31; 7, -32; 8. -35; 9. -36; 10, -37; 11, -38; 12 -39; 13, -40; 14, -41; 15, -42; 16, -43; 17, -44; and T, negative control) were plated onto 140 mm diameter NZY agar dishes and tested for serum reactivity. Serum from healthy donor was used in this panel.

Figure 2. Quantitative real-time RT-PCR analysis of *MYL6B*, *NUP107*, *HSPA4*, *MET,* and *SCOC* mRNA in adult normal tissues. Relative expression to testis was indicated.

Figure 3. Quantitative real-time RT-PCR analysis of *MYL6B*, *NUP107,* and *HSPA4* mRNA in 5 lung cancers and the adjacent normal tissues. T and N denote lung cancer and normal tissue, respectively. The mRNA expression levels of the cancer tissues were normalized to those of the respective adjacent normal tissues.

Table(s)

Table 1. SEREX-defined genes identified by screening of a lung adenocarcinoma cell line OU-LU-11 by autologous serum

Antigen	No. of clones	Identity/similarities	SEREX Dadabase search[1]	cDNA microarray (fold change)[2]				
				OU-LU-6	-11	-14	-17	-26
OY-LC-17	1/21	ISYI splicing factor homolog (S. cerevisiae) (***ISYI***)	0	1.61	2.56	1.51	2.37	0.71
OY-LC-18	1/21	Chromosome 9 open reading frame 23 (***C9orf23***)	1	1.16	0.64	1.85	1.21	1.82
OY-LC-19	3/21	Drebrin-like (***DBNL***), transcript variant 2	2	0.96	1.43	1.77	2.67	2.90
OY-LC-20	1/21	MORC family (W-type zinc finger 2) (***MORC2***)	0	1.63	0.90	2.04	1.42	1.87
OY-LC-21	1/21	Cytospin A (***CYTSA***)	0	-	0.59	-	0.48	-
OY-LC-22	1/21	WW, C2 and coiled-coli domain containing 2 (***WWC2***)	0	0.79	0.66	0.46	0.15	0.18
OY-LC-23	1/21	SAR1 gene homolog A (S. cerevisiae) (***SAR1A***)	0	1.78	5.24	1.12	3.11	0.95
OY-LC-24	1/21	Myosin, light polypeptide 6B, alkali, smooth muscle and non-muscle (***MYL6B***)	0	2.13	2.19	2.82	2.59	2.72
OY-LC-25	2/21	Polymerase (DNA directed), delta 2, regulatory subunit 50kDa (***POLD2***)	0	1.02	0.72	1.62	1.75	1.69
OY-LC-26	1/21	Utropin (***UTRN***)	0	0.78	0.94	0.46	0.54	1.27
OY-LC-27	1/21	Meckel syndrome, type 1 (***MKS1***)	0	1.58	0.68	0.98	1.69	2.35
OY-LC-28	2/21	Serologically defined colon cancer antigen 8 (***SDCCAG8***)	4	3.03	0.91	1.90	1.73	2.11
OY-LC-29	1/21	SWAP 70 protein (***SWAP70***)	16	1.25	2.15	1.28	0.86	0.90
OY-LC-30	1/21	Ubiquitin conjugating enzyme E2I (UBC9 homolog, yeast) (***UBE2I***)	0	1.72	0.93	2.65	1.98	2.50
OY-LC-31	1/21	Tight junction protein 2 (zona occludens 2 (***TJP2***), transcript variant 1	0	0.82	0.93	0.88	0.32	0.74
OY-LC-32	1/21	Multi PDZ domain protein (***MPDZ***)	0	1.43	1.34	0.47	1.02	0.62
OY-LC-33	1/21	Met proto-oncogene (hepatocyte growth factor receptor) (***MET***)	0	5.96	2.70	1.77	1.47	1.61
OY-LC-34	1/21	Ring finger protein, LIM domain interacting (***RLIM***), transcript variant 1	0	1.26	1.93	1.03	1.76	1.00
OY-LC-35	1/21	SAM domain, SH3 domain and nuclear localization signals 1 (***SAMSN1***)	0	0.14	0.88	0.19	0.18	0.20

[1]Number of clones reported at SEREX database (http://ludwig-sun5.unil.ch/CancerImmunomeDB/)

[2]Gene expressions in 5 adenocarcinoma cell lines were compared with normanl lung tissues by cDNA microarray analysis.

Table(s)

Table 2. SEREX-defined genes identified by screening of a lung adenocarcinoma cell line OU-LU-17 by autologous serum

Antigen	No. of clones	Identity/similarities	SEREX Dadabase search[1]	cDNA microarray (fold change) [2]				
				OU-LU-6	-11	-14	-17	-26
OY-LC-36	5/20	Nucleoporin 107 (***NUP107***)	1	1.63	1.70	3.47	4.23	1.59
OY-LC-37	4/21	Chromosome 21 open reading frame 58 (***C21orf58***)	0	0.28	1.01	0.54	0.48	0.72
OY-LC-38	3/20	Unknown	0	-	-	-	-	-
OY-LC-39	2/20	SEC63 homolog (S. cervisiae) (***SEC63***)	0	4.97	1.11	2.82	1.85	2.05
OY-LC-40	2/20	Tumor protein, translationally-controlled 1 (***TPT1***)	3	0.64	0.58	0.81	0.41	0.44
OY-LC-41	1/20	Heat shock 70kDa protein 4 (***HSPA4***)	6	2.33	1.53	2.47	1.49	2.72
OY-LC-42	1/20	Baculoviral IAP repeat-containing 2 (***BIRC2***)	3	1.89	1.07	1.02	0.75	0.69
OY-LC-43	1/20	Ribosomal protein S15A (***RPS15A***)	1	1.73	0.25	2.24	1.31	1.05
OY-LC-44	1/20	Short-coiled-coil protein (***SCOC***)	0	3.59	1.29	4.23	2.00	3.09

[1]Number of clones reported at SEREX database (http://ludwig-sun5.unil.ch/CancerImmunomeDB/)

[2]Gene expressions in 5 adenocarcinoma cell lines were compared with normanl lung tissues by cDNA microarray analysis.

Table(s)

Table 3. Reactivity of sera from healthy donors and lung cancer patients with SEREX-defined antigens

	Sera	
Antigen	Healthy donors	Lung cancer patients
OY-LC-17	4/10	3/10
OY-LC-19	6/10	3/10
OY-LC-23	2/10	2/10
OY-LC-25	2/10	1/10
OY-LC-28	3/10	2/10
OY-LC-31	3/10	3/10
OY-LC-32	1/10	1/10
OY-LC-35	5/10	2/32
OY-LC-36	4/27	4/32
OY-LC-37	6/27	3/32
OY-LC-38	5/27	3/32
OY-LC-39	4/27	4/32
OY-LC-40	11/27	2/32
OY-LC-41	8/27	1/32
OY-LC-42	6/27	1/32
OY-LC-43	9/27	1/32
OY-LC-44	6/27	2/32

Figure 1

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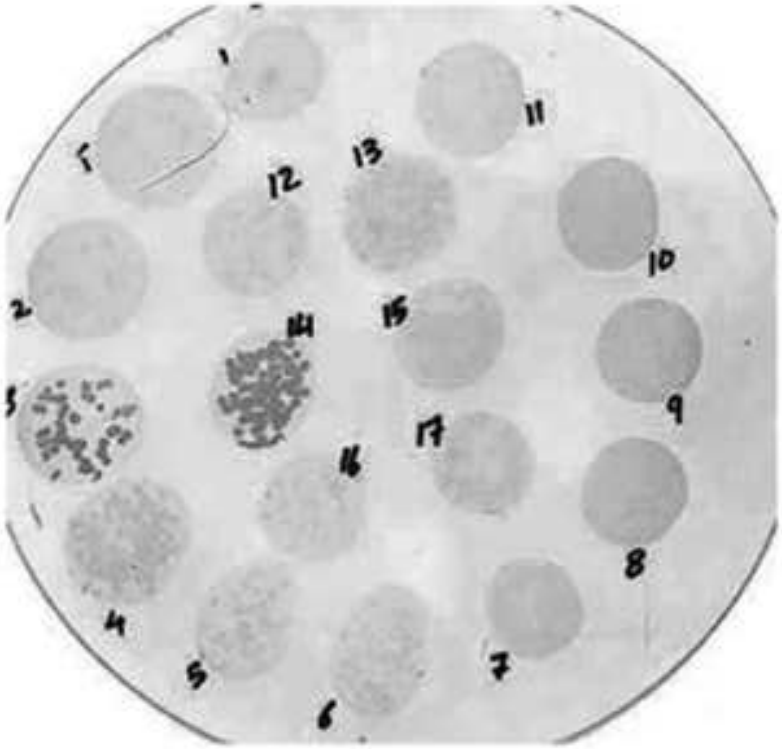

Figure 2

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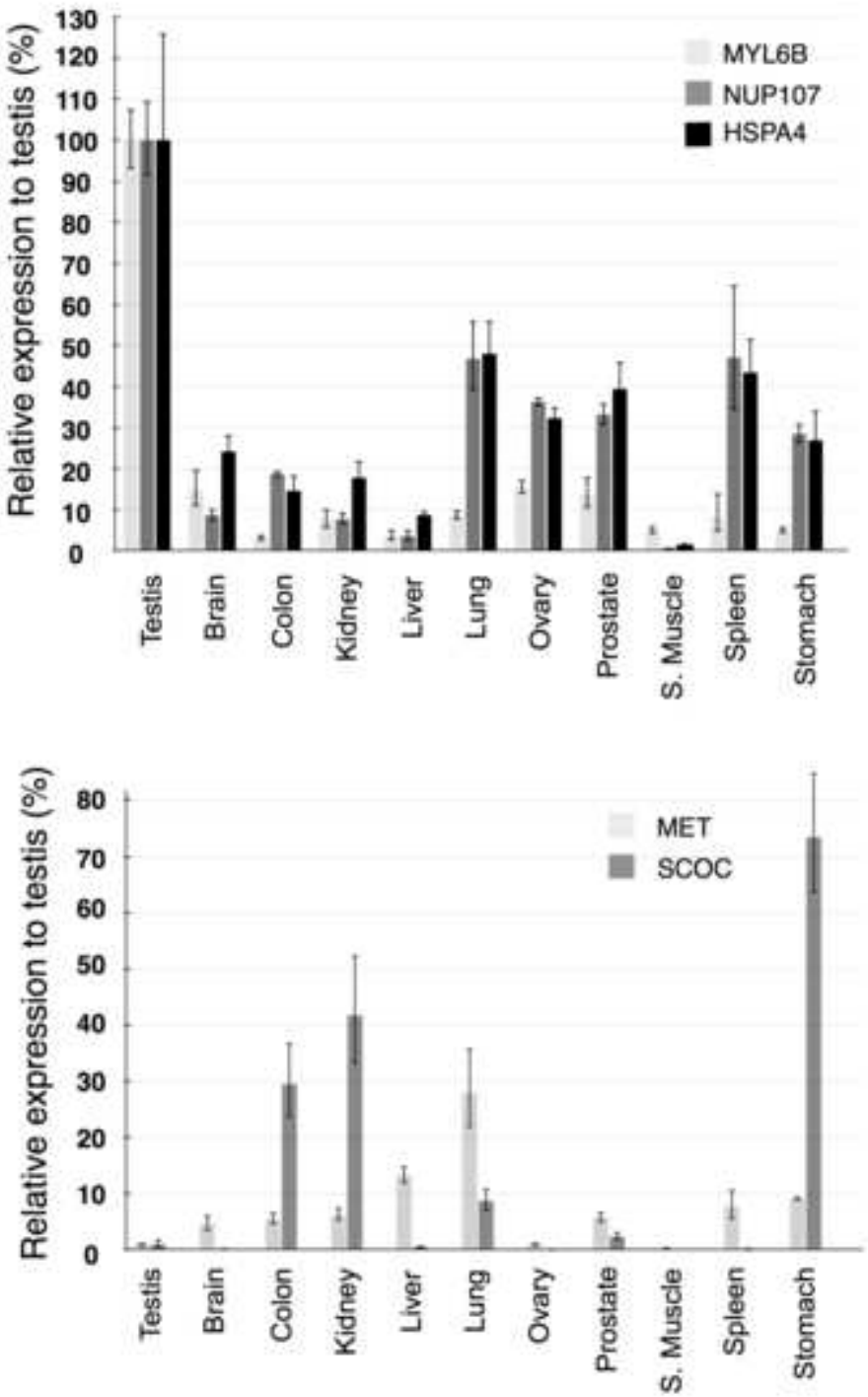

Figure 3

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