Identification of cell surface marker candidates on SV-T2 cells using DNA microarray on DLC-coated glass

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Abstract

We analyzed gene expression profiles of normal mouse fibroblast BALB/c 3T3 cells and its SV40 transformant SV-T2 cells using our originally developed cell surface marker DNA microarray, which is prepared on a diamond-like carbon-coated glass. As a result, CD62L and IL-6 receptor α gene expressions were upregulated in SV-T2 and were thought to be candidates for cell surface markers of the cells. The result of microarray analysis was validated by real-time quantitative PCR, immunohistochemistry and biological assays. These data show that our cell surface marker DNA microarray should be useful in finding the candidates of cell type-specific surface markers.
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Cell surface proteins are involved in biological functions, such as cell surface receptors, growth factor receptors, and cell adhesion molecules. Further, it has been found that there is a difference in their gene expression between tumor and corresponding adjacent normal tissue [1]. This heterogeneity of cell surface protein expression makes these molecules useful markers for specific tumor targeting [2–6]. Therefore, it would be extremely desirable to distinguish the cells or tissues in some diseases from those in a normal state by cell or tissue-specific surface markers.

To identify cell-specific surface markers, DNA microarray technology would be the best candidate, since large-scale, high-throughput screening of the expressing genes are possible at one time, whereas Northern hybridization or RT-PCR procedures are designed only for the characterization of the target genes.

The use of DNA microarray to determine gene expression has grown significantly during the last 10 years. Sophisticated data analysis has already demonstrated that various tumor types can be distinguished on the basis of their gene expression patterns, and a large number of genes are overexpressed in tumor compared to their normal tissue counterparts [7]. However, it still appears difficult to show reliable and reproducible results, depending on the density and the method of immobilization of spotted probe DNAs. In view of this, we employed diamond-like carbon (DLC)-coated glass to design an original DNA microarray. Since DLC coating can provide a high density of activated carbon atoms to covalently conjugate DNAs, reliable results could be expected.

The probe design should also be very carefully organized because various kinds of heterogeneity are present

by alternative splicing even in a single gene [8]. In many cases, gene families contain a wide expansion of molecules so that various designs of probes of genes should be used to expect the results in high precision. Instead of using as many probes as possible, another idea would be to organize a microarray with probes in a category, such as cell surface markers. Simultaneously, artificial RNA and its complementary DNA would be helpful to verify the procedure, if the sequence has no homology to any described human, mouse, or rat genes.

As a result, we designed an original DNA microarray and tried to characterize normal mouse fibroblast BALB/c 3T3 cells and its SV40 transformant SV-T2 cells.

Materials and methods

Reagents and Antibodies. IL-6 was from Wako Pure Chemical Industries (Japan). Anti-EGFR, anti-CD11b (anti-integrinαM) and anti-IL-6Rα antibodies were from Santa Cruz Biotechnology (CA). Anti-CD62L was from Southern Biotechnology Associates (AL). Anti-IL-6Rα neutralizing antibodies were from Innogenetics (Belgium).

Cell lines and cell cultures. Both mouse cell lines, BALB/c 3T3 and SV-T2, were maintained in DMEM supplemented with 10% (v/v) fetal bovine serum (FBS). For RNA isolation, SV-T2 cells were cultured on 0.5% bacto-agar (BD, NJ) to form colonies, while BALB/c 3T3 cells were cultured in a 100 mm dish.

Design of DNA microarray. We designed a DNA microarray that carries 60-mer oligonucleotide probes for 378 mouse cell surface proteins. This design of probes was implemented primarily to include the DNA sequence coding the transmembrane region or the GPI-anchor attachment site, which is the essential information for proteins to localize on the cell surface. The probes were synthesized with NH_2 radical at the 5′ end and spotted onto a DLC-coated glass slides, Gene Slide (Toyo Kohan, Japan). The surface of the Gene Slide was activated in advance at the carbon atoms and was ready to be conjugated with DNA by a covalent bond. The probes were spotted in quadruplicate, so that the intensity of signals for each gene could be analyzed statistically. This microarray included control spots complementary to the control RNA to verify coupling and hybridization described below.

RNA preparation. Total RNA was isolated from cells with the RNeasy Mini kit (Qiagen, Germany). After DNase treatment, the purity of RNA was assessed by the measurement of absorbance at 260 and 280 nm as the ratio of $OD_{260/280}$ at more than 1.4. Also, the integrity of RNA was determined by gel electrophoresis as the ratio of 28S/18S at more than 1.8. Genomic DNA contamination was tested by PCR with primers for GAPDH to amplify no visible product after 35 cycles.

Control RNA preparation. For in vitro synthesis of control RNA, we inserted a 756-bp fragment derived from EGFP cDNA sequence (GenBank Accession No. U76561) together with a poly(A)$_{24}$ downstream of the T7 promoter to construct a plasmid pBO795. The linear template for in vitro transcription with T7 RNA polymerase was prepared using the plasmid by PCR. The transcription reaction took place at 37 °C for 4 h. After DNase treatment and purification, the purity of the control RNA was assessed by measurement of absorbance at 260 and 280 nm and tested by PCR for template DNA as described in "RNA preparation."

DNA microarray assay. Twenty micrograms of total RNA from cells, together with 2 ng of control RNA, was used as the template of cDNA synthesis. Fluorescent-labeled cDNAs were prepared by reverse transcription of the mixtures in the presence of amino-allyl-dUTP

followed by coupling of the Cy3 dye (Amersham Biosciences). The labeled cDNA was purified with the QIAquick PCR Purification Kit (Qiagen) and then used for hybridization at 55 °C for 15 h. The slides were washed and scanned using an FLA8000 scanner (Fuji Film, Japan). The intensity of each signal was analyzed with GenePix Pro 5.1 software (Axon). Gene expression levels were compared to one another by relative fluorescent intensity (RFI), where RFI is the percentage of the fluorescent intensity of each gene and considering that of the internal control to be 100%.

Real-time quantitative PCR. A half nanogram of internal control RNA was mixed with 5 μg of total RNA from each cell line, and reverse-transcribed for single-strand cDNAs using oligo(dT)$_{18}$ primer. Then real-time quantitative PCR (RT-qPCR) was performed using Light Cycler DX400 (Roche) with LightCycler FastStart DNA Master SYBR Green I kit (Roche) in triplicate. The PCR was 40 cycles of 10 s at 95 °C, 10 s at 60 °C, and 25 s at 72 °C. The melting curve analysis was 0 s at 95 °C, 15 s at 65 °C, up to 98 °C, and cooled to 40 °C. The primer pairs used were as follows: 5′-GCCCCAGTGTCAGTAT GTGGT-3′ and 5′-GGGATGAATGAGCGAGGGGAA-3′ for CD62L, 5′-GCAGTTCCAGCTTCGATACCG-3′ and 5′-GTCATAA GGGCTCTGTGCGTC-3′ for IL-6Rα, 5′-CAGGGAGTGCGTGGA GAAATG-3′ and 5′-GCTCCCGAACCCAGAACTTTG-3′ for EGFR (GenBank Accession No. NM_007912), 5′-TGGAGCTGCCTGTG AAGTACG-3′ and 5′-TTACTGAGGTGGGGCGTCTTG-3′ for CD11b.

Immunofluorescence. Cells cultured in Lab-Tek chamber slides were washed with phosphate-buffered saline (PBS), fixed with 10% formalin and permeabilized with 0.2% Triton X-100 on the glass slides. Cells were then incubated with the primary antibody of each protein and second antibody IgG-FITC. Fluorescence images were collected on a Zeiss confocal microscope and photographed using a 63× immersion objective.

Cell growth assay. The cells were plated in 96-well plates. IL-6 and/or anti-IL-6Rα were added daily to maintain the concentration for 5 days. Cell growth was determined by 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [9]. The relative cell growth for each treatment is expressed as a percentage of untreated control cultures. The statistical significance of differences between the means of six replicates was assessed by Student's t test, with $P < 0.05$ being considered significant.

Results and discussion

Gene expression profiling of BALB/c 3T3 and SV-T2 cells

In order to screen cell surface marker proteins differentially expressed in the two cell lines, we performed DNA microarray analysis. The results of our original mouse cell surface marker arrays on DLC-coated glass are summarized in Fig. 1. The scatter plot of the gene expression pattern between independent experiments on BALB/c 3T3 cells shows excellent reproducibility of the results with a correlation coefficient of 0.99 (Fig. 1A). When BALB/c 3T3 cells were compared to SV-T2 cells, no difference in the expression levels of almost 200–250 of 378 genes was found between the two cell lines. We chose 19 genes whose expression was considered significantly upregulated in SV-T2 cells when compared with BALB/c 3T3 cells, judging by the average difference in value of relative fluorescence intensity (RFI) greater than 20 (Fig. 1B, Table 1). In a similar
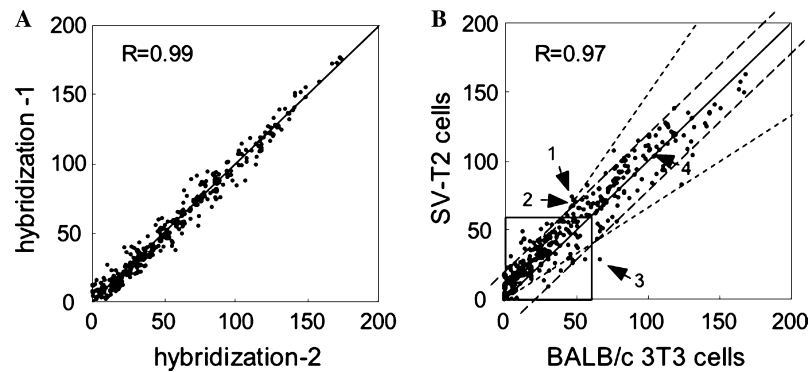
Fig. 1. Scatter plots of gene expression pattern. (A) Two independent hybridizations of BALB/c 3T3 cells are compared. (B) SV-T2 cells vs BALB/c 3T3 cells. The cutoffs for average difference values of relative fluorescence intensity (RFI) greater than 20 are indicated by dashed lines and 1.5-fold upregulated and downregulated are indicated by short dashed lines. Each arrowhead corresponds to the genes extracted in this study. (1) CD62L; (2) IL-6Rα; (3) CD11b; (4) EGFR.

Table 1
Genes significantly upregulated in SV-T2 cells

Gene	GenBank Accession	A[a]	B[a]	d_1[b]	t_1[c]
Asialoglycoprotein receptor 2	NM_007493	70.6	91.1	20.5	1.3
interleukin 12 receptor, beta 1	NM_008353	20.2	40.7	20.6	2.0
adrenergic receptor, beta 2	X15643	1.9	22.6	20.8	12.1
gap junction membrane channel protein alpha3	NM_016975	33.1	53.8	20.8	1.6
prostaglandin D receptor	NM_008962	49.3	70.3	21.0	1.4
neuromedin B receptor	NM_008703	93.2	114.6	21.4	1.2
interleukin 6 receptor, alpha (IL-6Rα)	X53802	48.8	71.0	22.3	1.5
integrin alpha 6	X69902	13.8	36.1	22.4	2.6
gap junction membrane channel protein beta 6	NM_008128	17.4	41.6	24.3	2.4
tumor-associated calcium signal transducer 1	NM_008532	57.7	82.4	24.6	1.4
contactin 3	NM_008779	29.3	54.3	25.1	1.9
glycophorin A	NM_010369	25.0	50.4	25.4	2.0
occludin	U49185	4.0	30.8	26.8	7.7
selectin, lymphocyte (CD62L)	NM_011346	46.7	74.2	27.5	1.6
platelet/endothelial cell adhesion molecule	L06039	11.1	40.5	29.4	3.6
glycoprotein 5 (platelet)	NM_008148	13.7	43.1	29.5	3.2
integrin beta 1 (fibronectin receptor beta)	Y00769	13.6	43.4	29.8	3.2
neuropeptide Y receptor Y6	NM_010935	76.5	107.2	30.7	1.4
interleukin 1 receptor, type I	M27960	12.2	48.5	36.3	4.0

[a] A and B denote RFIs in BALB/c 3T3 and SV-T2 cells, respectively.
[b] $d_1 = B - A$.
[c] $t_1 = B/A$.

Table 2
Genes significantly downregulated in SV-T2 cells

Gene	GenBank Accession	A[a]	B[a]	d_2[b]	t_2[c]
Burkitt lymphoma receptor 1	NM_007551	123.7	82.7	20.5	1.3
integrin alpha M (CD11b)	X07640	66.3	28.4	20.6	2.0
galanin receptor 1	Y15004	56.4	28.5	20.8	12.1
histamine receptor H 1	NM_008285	131.3	104.1	20.8	1.6
prostaglandin E receptor EP2 subtype	NM_008964	78.2	55.8	21.0	1.4
coagulation factor III	NM_010171	30.6	8.9	21.4	1.2
purinergic receptor P2X-like 1, orphan receptor	NM_011028	47.2	25.9	22.3	1.5
intercellular adhesion molecule	M31585	64.3	43.2	22.4	2.6

[a] A and B denote RFIs in BALB/c 3T3 and SV-T2 cells, respectively.
[b] $d_2 = A - B$.
[c] $t_2 = A/B$.

manner, eight downregulated genes are listed in Table 2. In order to pick up the candidates of the potential cell surface markers for ST-T2 cells, we tentatively applied the following two criteria to the 19 genes: (1) a ratio value of RFI in SV-T2 versus RFI in BALB/c 3T3 greater than 1.5, i.e., B/A ⩾ 1.5, where A and B represent RFI in BALB/c 3T3 and SV-T2 cells, respectively; (2) a value of RFI in SV-T2 greater than 60, i.e., B > 60, which is equivalent to the expression level of a housekeeping gene, GAPDH. Among the criteria, the first represents a significant increase, while the second represents an expression level that should be useful as an actual cell surface marker. Only two genes, IL-6Rα and CD62L, satisfied the criteria. Through inverse comparison, three genes, CD11b, Burkit lymphoma receptor 1 and intercellular adhesion molecule, satisfied the criteria (Table 2). Since CD11b showed the highest ratio of A/B and as the antibody was commercially available, further validation of this gene was carried out as the representative of the downregulated expression.

Confirmation of differentially expressed genes by real-time quantitative PCR

Real-time quantitative PCR analyses were used to validate top ranks of differentially expressed genes profiled by the microarray. IL-6Rα and CD62L were 11- and 300-fold upregulated in SV-T2 cells, respectively, while CD11b was less than 0.5-fold downregulated (Table 3). The level of CD11b expression in SV-T2 cells, which required more than 40 cycles of PCR amplification to be confirmed, was so low that the precise estimation of downregulation was difficult to accomplish by this method. As for the expression of the EGF receptor (EGFR) gene, the RFI of BALB/c 3T3 cells was 101.7 and that of SV-T2 cells was 108.3, which made the spot close to the 45° diagonal line in the scatter plot (Fig. 1B). We considered that the levels are almost equivalent and took this gene as representative of unchanged expression. As a result, our DNA microarray appears to be able to successfully detect expression change more than 10-fold.

Detection of candidates for cell surface markers by immunofluorescence

Next, we raised two questions of (a) whether the corresponding proteins are localized on the surface of the

Table 3
Summary of RT-qPCR on the change in gene expression

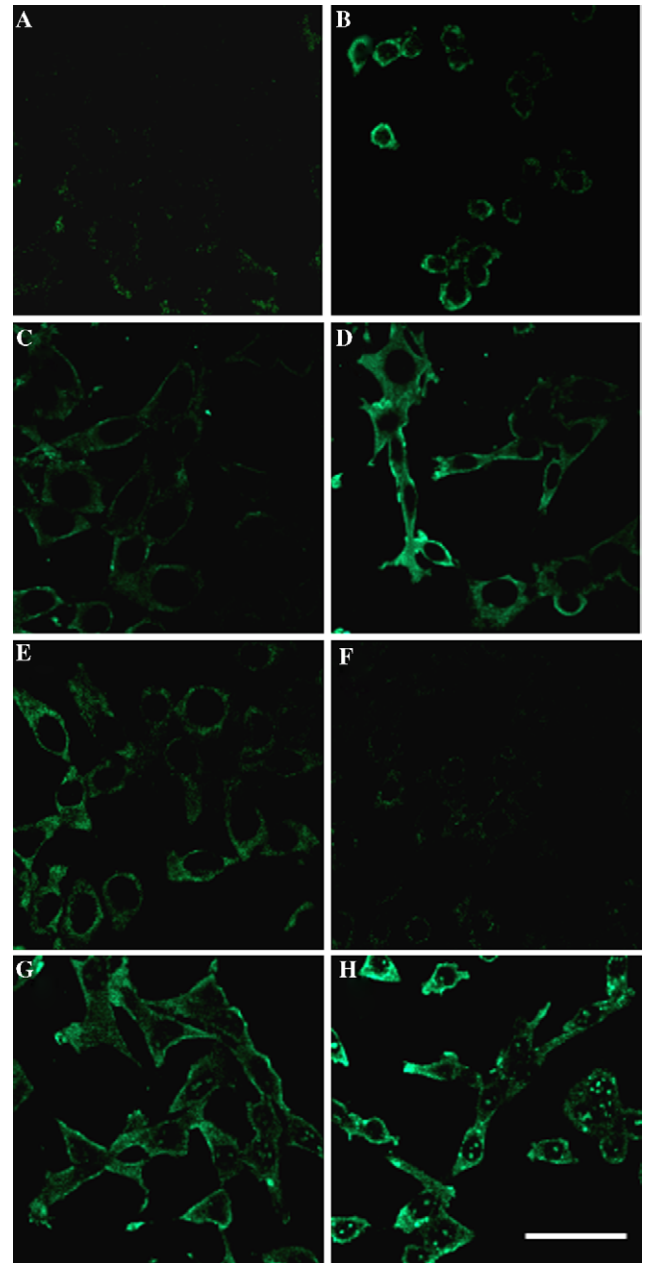| Gene	SV-T2 cells relative to BALB/c 3T3 cells (folds)
CD62L	300.2 ± 23.6
IL-6Rα	10.8 ± 2.0
CD11b	0.5 ± 0.3
EGFR	4.4 ± 0.4

Fig. 2. Detection of cell surface proteins by immunofluorescence. BALB/c 3T3 cells (A,C,E,G) and SV-T2 cells (B,D,F,H) were immunostained with antibodies against CD62L (A,B), IL-6Rα (C,D), CD11b (E,F), and EGFR (G,H). Scale bar = 50 μm.

cells, and (b) whether the relative amount of the proteins on the two cell lines reflected the level of gene expression. We evaluated the protein expression of the four cell surface proteins by immunofluorescence (Fig. 2). In BALB/c 3T3 cells there was no positive staining or only faint staining for IL-6Rα and CD62L, while SV-T2 cells showed strong fluorescence. CD11b showed inverse results. EGFR were detected on the surface of both cell lines. These results are consistent with the results obtained by the microarray and RT-qPCR.

SV-T2 cells respond to IL-6 via IL-6Rα

Since we found that IL-6Rα was increased in SV-T2 cells, we assessed the sensitivity of these cells to IL-6. IL-6 is a pleiotropic cytokine that is implicated in a variety of cellular functions in immune, hematopoietic, neural, and hepatic systems. IL-6 has also been shown to influence the proliferation of normal and tumor-derived cells [10]. When the cells were treated with IL-6, the proliferation of SV-T2 cells was inhibited in a dose-dependent manner, while no effects were observed in BALB/c 3T3 cells (Fig. 3). IL-6 acts through the receptor complex that consists of an α chain subunit (i.e., IL-6Rα) with specific affinity for IL-6 and a β chain subunit of signal transducer known as gp130 without specific affinity to
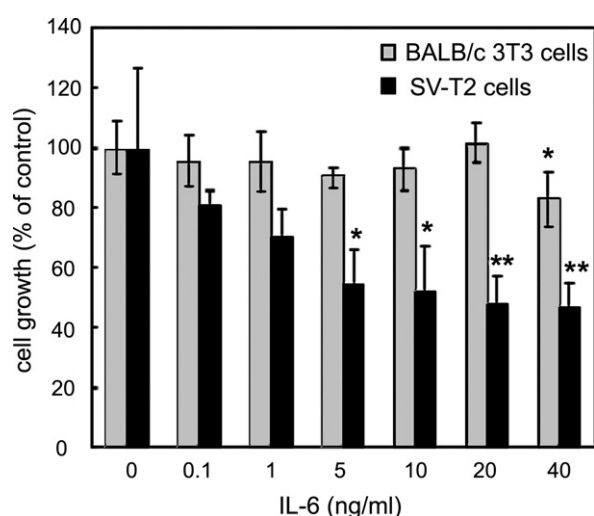

Fig. 3. Effect of IL-6 on the growth of BALB/c 3T3 and SV-T2 cells. Statistical significance, compared with that of IL-6 non-treated cells, is indicated by $*P < 0.05$ and $**P < 0.01$.
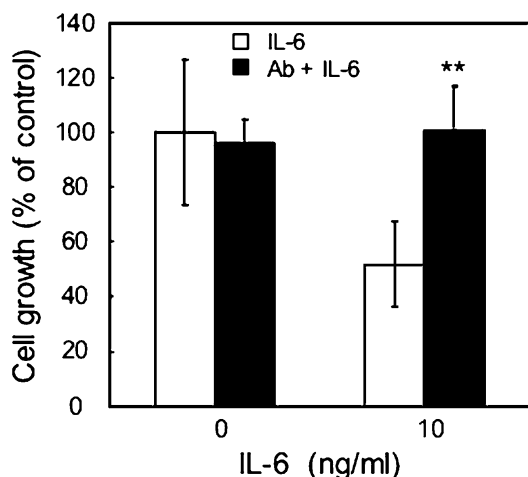

Fig. 4. Effect of anti-IL-6Rα antibodies on IL-6-treated SV-T2 cells. Cells were stimulated with 10 ng/ml of IL-6 in the presence, or absence, of anti-IL-6Rα and cultured for 5 days. Statistical significance, compared with that of IL-6 treated cells, is indicated by $**P < 0.01$.

ligands [10,11]. RT-qPCR showed the expression of IL-6Rα was 11-fold increased in SV-T2 cells compared to BALB/c 3T3 cells, while that of gp130 was almost equivalent between the two cell lines (approximately 1.4-fold). Anti-IL-6Rα antibodies neutralized the IL-6-induced inhibition of proliferation in SV-T2 cells (Fig. 4). Thus we concluded that the inhibition was IL-6Rα-dependent. In this context, SV-T2 cells might acquire sensitivity to IL-6 via upregulated IL-6Rα.

Conclusion

The cell surface marker array developed on the DLC-coated glass slide has been successfully designed to carry the 378 oligo DNA probes for independent cell surface proteins. This microarray was employed here in profiling the gene expression of BALB/c 3T3 and SV-T2 cells. As a result, CD62L and IL-6Rα were found to be the candidates of the significantly upregulated genes in SV-T2 cells. RT-qPCR, immunohistochemical analysis and biological function analysis supported the result of microarray analysis. CD62L and IL-6Rα are proposed as the candidates of cell surface markers in SV-T2 cells. These data highlight the power of a DNA microarray with high reproducibility using the novel platform of a DLC-coated glass slide to identify cell surface markers, which might serve as molecular targets for the diagnosis or treatment of diseases. It is expected to expand the number of genes to cover all the cell surface markers for the arrays.

Acknowledgments

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