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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.

Keywords: Cell surface marker; DNA microarray; DLC-coated glass; BALB/c 3T3 cells; SV-T2 cells; IL-6 receptor α

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

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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′-GCCCCAGTGTCAGTATGTGGT-3′ and 5′-GGGATGAATGAGCGAGGGGAA-3′ for CD62L, 5′-GCAGTTCCAGCTTCGATACCG-3′ and 5′-GTCATAAGGGCTCTGTGCGTC-3′ for IL-6Rα, 5′-CAGGGAGTGCGTGGAGAAATG-3′ and 5′-GCTCCCGAACCCAGAACTTTG-3′ for EGFR (GenBank Accession No. NM_007912), 5′-TGGAGCTGCCTGTGAAGTACG-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