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Profiling of Gene Expression in Human Keratinocyte Cell Line Exposed to Quantum Dot Nanoparticles

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Abstract

Quantum Dot (QD) nanoparticles are used in various industrial applications, such as diagnostic, drug delivery, and imaging agents of biomedicine. Although QDs are extensively used in many medical science, several studies have been demonstrated the potential toxicity of nanoparticles. The first objective of this study was to investigate the nanotoxicity of QDs in the HaCaT human keratinocyte cell line by focusing on gene expression pattern. In order to evaluate the effect of QDs on gene expression profile in HaCaT cells, we analyzed the differential genes which related to oxidative stress and antioxidant defense mechanisms by using human cDNA microarray and PCR array. A human cDNA microarray was clone set, which was sorted for a list of genes correlated with cell mechanisms. We tried to confirm results of cDNA microarray by using PCR array, which is pathway-focused gene expression profiling technology using Real-Time PCR. Although we could not find the exactly same genes in both methods, we have screened the effects of QDs on global gene expression profiles in human skin cells. In addition, our results show that QD treatment somehow regulates cellular pathways of oxidative stress and antioxidant defense mechanisms. Therefore, we suggest that this study can enlarge our knowledge of the transcriptional profile and identify new candidate biomarker genes to evaluate the toxicity of nanotoxicology.

Keywords: Quantum dot, cDNA microarray, PCR array, Keratinocyte

Skin is a potential route of exposure to various materials, like especially nano materials. Although there are many hydrophobic layers in human skin which limit absorption of most molecules as the skin barrier, the permeability of the skin to these nano particlels is still unknown[1]. For these reasons, it is important to study for the cytotoxic effects of nano particles in human skin and estimate a dose of these materials in safe range.

Quantum dot (QD) nanoparticles are well known for their special characteristic of strong fluorescence, and therefore are used in various industrial applications or therapeutic applications such as diagnostic drug delivery and imaging agents of biomedicine[2-6]. QDs are commercially available in various chemical compositions, shape and sizes and are usually multilayered. QD's center is constituted by a heterogeneous colloidal core of several inorganic atoms, and its surface is coated in one or more layers, such as the use of increasing solubility in a biologically compatible medium[7]. Because QDs have special abilities like intense and photostable fluorescence unlike other nanostructures, they can be applied for different applications, such as bioconjugation to antibodies or specific ligands of receptors for drug delivery[2]. Thus, we used QDs as promptly accessible tools to determine the permeability of skin to nanostructures with several physicochemical properties which have important associations for nano materials risk assessment. However, despite their wide use, several studies have demonstrated the potential toxicity of nanoparticles[8-11], so before QDs can be used safely in humans (e.g., cosmetics commercial products, medicines and so on), we need to know more information about their potential for toxicity and interactions or mechanisms in biological systems. Several studies have reported that the QD surface coatings and charge can influence the toxicity of QD[12,13] because novel properties of sizes and shapes of nano materials may have consequences for toxicology[14]. So, we selected commercial QDs of

two core/shell sizes and shapes that are carboxylic acids surface coatings and polyethylene glycol (PEG)-amine surface coatings. The carboxylic acids and PEG-amine surface coatings were known to reduce non-specific binding to several types of cells and enhance QD's stability and water-solubility[15-17]. Despite various research about nano materials, there are few studies focused on human skin cells. For this reason, we have studied the nanotoxicity of QDs in human keratinocyte cells (HaCaT) by a molecular approach in nanotoxicology research. In order to evaluate QD's cytotoxicity and inflammation potential, we have monitored gene expression in HaCaT cells treated QDs by using human cDNA Microarray that was derived from a commercially available master set of about 15,000 human verifies-sequences (Research genetics, Inc, AL, USA). This cDNA clone set that was sorted for a list of genes involved 1,152 elements, representing families, correlated with molecules related to cell growth and maintenance, differentiation, development, proliferation, transformation, cell cycle progression, transcription, oncogenes, and especially immune response. Our PCR array has the differential expression of 96 genes which related to oxidative stress and antioxidant defense mechanisms. This RT^2 Profiler PCR Array System is the most reliable and accurate technique for analyzing the expression of pathway-focused or disease-specific genes[18,19]. We focused on especially gene expression changes due to nanoparticles-induced cytotoxicity in human skin cells.

Human cDNA Microarray of QDs Treated Cells

To identify the gene expression profiles which are

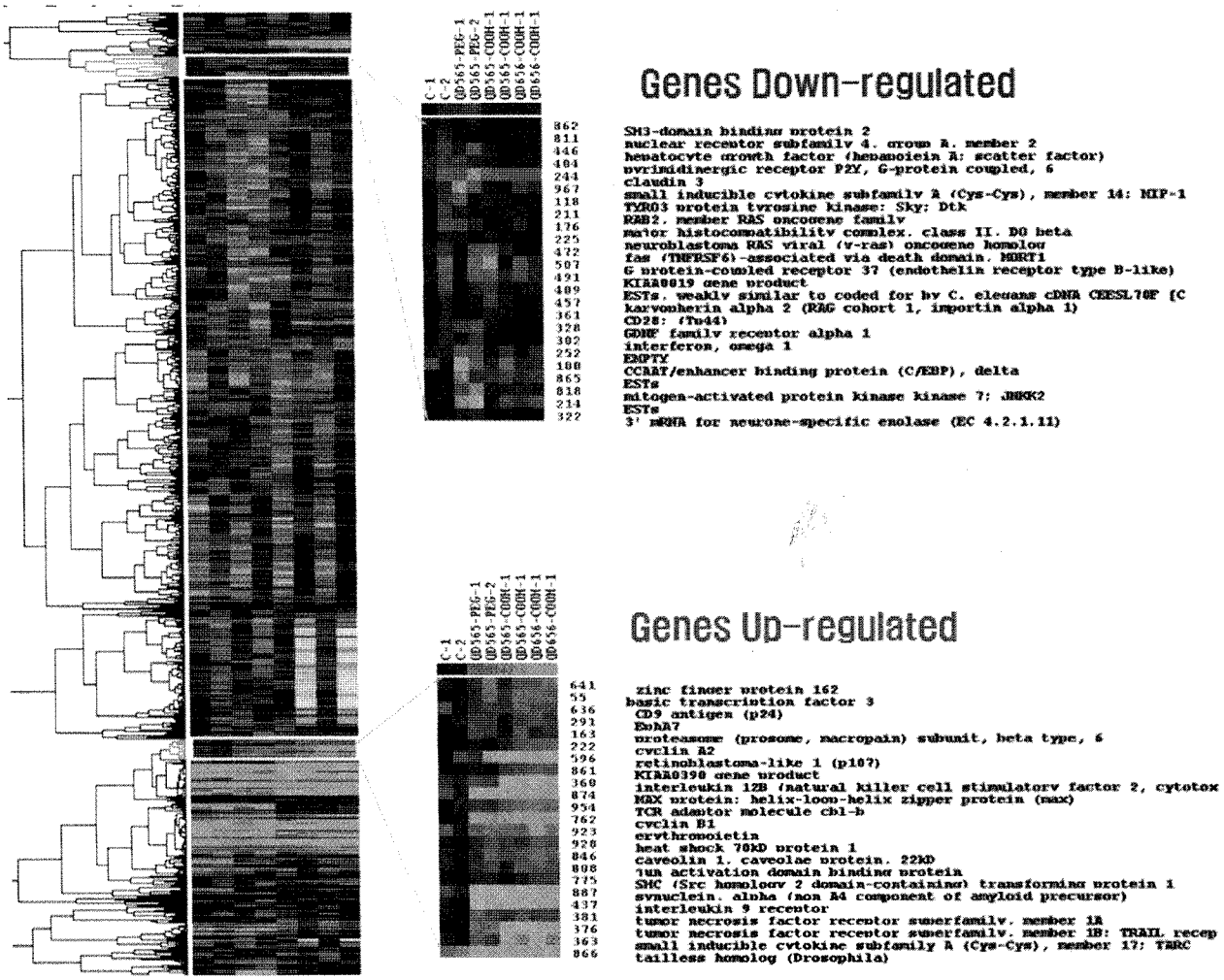

Figure 1. Up & down-regulated genes of HaCaT cells treated with QDs. HaCaT cells (1.5×10^4 cells/wells) were treated with 50 nM QDs for 12 hr. Microarray data from control group and experimental group were combined and clustered. Cluster analysis was performed on Z-transformed microarray data using two separate program available as shareware form Michael Eisen's lab.

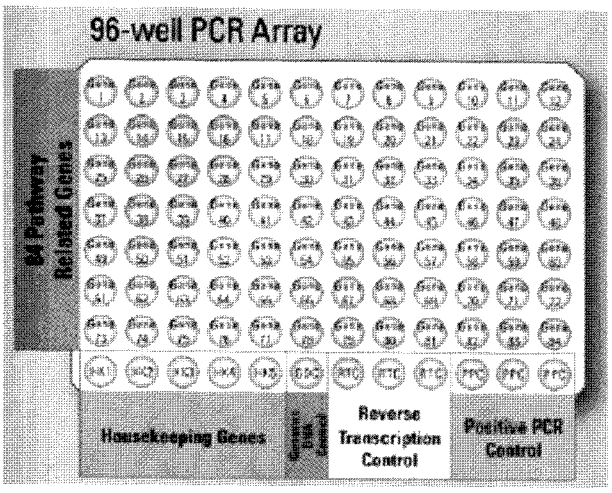

Figure 2. Layout of the cataloged pathway focused PCR arrays (96-well). Wells A1 through G12 contain a real-time PCR assay for genes from the same biological pathway or the same disease state or genes that are otherwise functionally-related. Wells H1 through H5 contain a housekeeping gene panel to normalize PCR Array data. Well H6 contains the Genomic DNA Control (GDC). Wells H7 through H9 contain replicate Reverse Transcription Controls (RTC). Wells H10 through H12 contain replicate Positive PCR Controls (PPC).

regulated by QDs, we performed the cDNA microarray and PCR array. The up- and down-regulated genes in QD treated HaCaT cells are listed in Tables 1, 2 and 4. Our microarray analysis showed that exposure of QD565-PEG, QD565-COOH and QD656-COOH up-regulated 23, 28 and 33 genes compared to non-treated cells. Especially, three types of QD commonly elevated mRNA expression of oxidative stress related genes such as nitric oxide synthase 2 (NOS2), prostaglandin-endoperoxide synthase (PTGS), superoxide dismutase 1 (SOD1), peroxidasin homolog (PXDN), thyroid peroxidase (TPO) and keratin 1 (KRT1). However, treatment of PEG and COOH in HaCaT cells down-regulated 27, 34 and 33 genes, respectively. We used a hierarchical clustering to show the relationships between the control group and experimental group, and visualized up- and down-regulated genes (Figure 1). Red color represented greater expression than the mean, green color represents less expression than one and black represented the median level.

RT² Profiler™ PCR Array of QDs Treated Cells

Our PCR array data demonstrated that genes related to oxidative stress or antioxidant defense mechanisms were differentially regulated by QDs compared with non-treated cells. Gene expression analyses showed up-regulated genes of 14, 31, 20 in QD565-PEG, QD565-COOH and QD656-COOH treated cells, and down-regulated genes of 8, 9, 7 in each group (data is not shown). For example, diacylglycerol kinase, kappa (DGKK), glutathione peroxidase (GPX), lactoperoxidase (LPO), phosphoinositide-binding protein (PIP3-E) and proteoglycan 3 (PRG3) were up-regulated, but metallothionein 3 (MT3), BCL2/adenovirus E1B interacting protein 3 (BNIP3), aldehyde oxidase 1 (AOX1) and angiopoietin-like 7 (ANGPTL7) were down-regulated by QDs treatment.

Discussion

To demonstrate that nano particles would have cytotoxicity when they are taken up in human skin, we used commercially available QDs. Since there are many studies which show that QD cytotoxicity depends on a variety of physicochemical properties such as size, shape and surface coatings, we used two sizes (QD 565 and QD 655) and two different surface coatings (PEG and carboxylic acids)[20,21].

The objective of this study was to investigate the nanotoxicity of QDs in HaCaT human keratinocyte cells focused on gene expression profiles. Our cDNA microarray and PCR array data showed that commonly increased genes were related to immune cytotoxicity, stress response and fibrosis synthesis. Moreover, the expression of genes associated with antioxidant defense mechanisms was decreased.

In human cDNA microarray, PTGS is the cyclooxygenase that acts both as a dioxygenase and as a peroxidase[28]. Small inducible cytokine subfamily (SCYE1) is the cytokine that is specifically induced by apoptosis, and it is involved in the control of angiogenesis, inflammation, and wound healing[29]. BCL2-like 11 (BCL2L11) acts as an apoptotic activator and can be induced by nerve growth factor (NGF) inducing neuronal and lymphocyte apoptosis[30]. In PCR array, LPO and ANGPTL7 act as oxidoreductase and peroxidase activity that is involved oxidation reduction and response to oxidative stress and PIP3-E also is an oxygen transporter and acts as a peroxidase[31].

Although both cDNA microarray and PCR array for analyzing the gene expression profiles showed many genes which were differentially regulated by QD treatment, the results of the two methods did not showed the correlation. We could not find the exact same genes that were up- or down-regulated by QD treatment. In this study, QD treatment was found to alter the expression of many genes involved in immune cytotoxicity to oxidation mechanisms. These observations led to us speculate that QDs exposure regulates global gene expression profiles and thereby increases oxidative stress in skin cells. Also, we suggest that

Table 1. Up & down regulated genes of HaCaT cells treated with QD-565-PEG.

Gene name	Z-ratio	Gene name	Z-ratio
MAX protein; helix-loop-helix zipper protein (max)	6.65	ESTs, weakly similar to eukaryotic initiation factor 4A-I	−4.68
P glycoprotein 1/multiple drug resistance 1; MDR1	6.36	interleukin 2 receptor, alpha	−4.03
gap junction protein, beta 2, 26 kD (connexin 26)	5.99	POU domain, class 6, transcription factor 1	−3.95
protein phosphatase 2, regulatory subunit B (B56), alpha isoform	5.87	adenosine A2a receptor	−3.90
ESTs, highly similar to mitogen-activated protein kkk kinase 2	5.77	Bruton agammaglobulinemia tyrosine kinase	−3.66
IMP (inosine monophosphate) dehydrogenase 1	4.74	ferritin, heavy polypeptide 1	−3.61
synuclein, alpha (non A4 component of amyloid precursor)	4.63	chemokine (C-C motif) receptor 5	−3.54
protein tyrosine phosphatase, receptor type, N; islet cell antigen ICA-512 mRNA	4.16	ESTs, highly similar to NADH-ubiquinone oxidoreductase PDSW subunit	−3.47
small inducible cytokine A5 (RANTES)	4.04	deoxyribonuclease II, lysosomal	−3.35
Muscarinic acetylcholine receptor M1 (human)	3.86	signal transducer and activator of transcription 2, 113 kD	−2.81
N-acylaminoacyl-peptide hydrolase	3.76	lymphocyte antigen 64 (mouse) homolog, radioprotective	−2.78
Interleukin 9 receptor	3.36	thyroid stimulating hormone receptor	−2.56
E2F transcription factor 3	3.33	Bcl-2-interacting killer (apoptosis-inducing)	−2.46
cyclin B1	2.99	receptor tyrosine kinase axl	−2.28
regulator of G-protein signalling 1	2.72	calmodulin 3 (phosphorylase kinase, delta)	−2.24
casein kinase 2, alpha 1 polypeptide	2.66	CD59 antigen p18-20 (Ag identified by monoclonal Abs	−2.19
MAD (mothers against decapentaplegic, Drosophila) homolog 3; Smad3	2.27	integrin, α V (vitronectin receptor, α-polypeptide, Ag CD51)	−2.18
neuroendocrine-specific protein C like (foocen); NOGO	2.24	S100 calcium-binding proteinA1 (annexin II ligand, calpactin I)	−2.15
tumor necrosis factor (ligand) superfamily, member 14; LTG	2.20	major histocompatibility complex, class II, DQ beta 1	−2.14
EAT; myeloid cell leukemia sequence 1 (BCL2-related)	2.03	heat shock transcription factor 4	−2.07
ribosomal protein S25	2.03	DNA (cytosine-5-) methyltransferase 3 beta	−2.06
pentaxin-related gene, rapidly induced by IL-1 beta	2.01	transcription factor AP-4 (activating enhancer-binding protein 4)	−2.03

this study can enlarge our knowledge of the transcriptional profile and identify new candidate biomarker genes to evaluate the toxicity of nanomaterials. Although several studies have demonstrated the potential toxicity of nanoparticles, QDs still have potential value in various industrial and biomedical applications, so we need more experimentation and information to fully explain the effects of these nanomaterials and whether they can be used safely in humans.

Materials & Methods

Quantum Dot (QD)s

Quantum dot 565 that has fluorescence emission maxima at 565 nm coated carboxylic acids and quantum dot 655 that has fluorescence emission maxima at 655 nm coated polyethylene glycol (PEG)-amine are spherical core/shell shape with a 4.6 nm core/shell diameter. Quantum dot 655 coated carboxylic acids is ellipsoid core/shell shape that measures 12 nm (major axis) × 6 nm (minor axis) in size (Table 3).

Cell Culture and QDs Treatment

Human Keratinocyte Cell Line, HaCaT, were cultured in 6-well plates in low calcium medium DMEM (Dulbecco Eagle's minimum essential medium, Biowhittaker, Belgium) with 10% fetal bovine serum, antibiotics (Penicillin 100 U/mL and Streptomycin 100 μg/mL, Invitrogen, Milano, Italy), 4 mM L-Glutamine, and supplemented with calcium chloride at 1.4 mM final concentration in a humidified atmosphere of 5% CO_2 at 37°C. HaCaT cells grown in this low calcium medium did not require addition of trypsin inhibitor in when assayed for differentiation[22]. Before QDs treatment, HaCaT cells were plated at 1.5-2.0 × 10^4 cells/cm^2 and were grown to 60-80% confluency. The QDs of different coatings are supplied at final concentration of 8.4 μM in 50 mM boric acid.

RT² Profiler™ PCR Array

To use RT² First Strand kit (Superarray, Fredrick, MD, USA), we prepared 15 μL total RNA from cells. The samples were used with RT² First Strand kit (Superarray, Fredrick, MD, USA) with the addition 5· gDNA elimination buffer. RT cocktail was added to each sample, then PCR was performed using refernce mixtures. After the amplification process, thermal cycling and fluorescence detection were analyzed by using an ABI 5700 Prism (PE Applied Biosystems, Foster City, CA, USA). Finally the changes of gene expression in our samples were analyzed according to the Web-Based PCR Array Data Analysis tool. This is summery to modified protocol for RT² Profiler™ PCR Array. Step 1 : Prepare cDNAs from RNA samples. Step 2 : Add cDNA to RT² qPCR master mix. Step 3 : Aliquot the mixture across PCR arrays. Step

Table 2. Up & down regulated genes of HaCaT cells treated with QD-565-COOH.

Gene name	Z-ratio	Gene name	Z-ratio
amyloid beta (A4) precursor protein (protease nexin-II)	7.22	ESTs, weakly similar to eukaryotic initiation factor 4A-I	−4.38
MAX protein; helix-loop-helix zipper protein (max)	7.16	Bruton agammaglobulinemia tyrosine kinase	−4.32
ESTs, highly similar to fibroblast growth factor 12	5.85	proteasome (prosome, macropain) subunit, alpha type, 1	−4.11
MAD (mothers against decapentaplegic, Drosophila) homolog 5; Smad5	4.92	singed (Drosophila)-like (sea urchin fascin homolog like)	−3.71
synuclein, alpha (non A4 component of amyloid precursor)	4.46	thyroid stimulating hormone receptor	−3.53
small inducible cytokine subfamily B (Cys-X-Cys), member 14 (BRAK)	4.21	ESTs, moderately similar to zinc finger protein ZNF49	−3.42
no match on BLAST search	3.82	chemokine (C-C motif) receptor 5	−3.38
apoptosis-associated tyrosine kinase	3.66	DNA (cytosine-5-) methyltransferase 3 beta	−3.27
retinoblastoma-like 1 (p107)	3.62	lymphocyte antigen 64 (mouse) homolog, radioprotective, 105 kD	−3.21
cyclin B1	3.17	integrin, alpha 9	−3.21
claudin 3	2.97	Bcl-2-interacting killer (apoptosis-inducing)	−3.21
tumor necrosis factor receptor superfamily, member 1B; TRAIL receptor	2.86	signal transducer and activator of transcription 2, 113 kD	−3.19
Interleukin 9 receptor	2.52	interleukin 2 receptor, alpha	−3.13
tailless homolog (Drosophila)	2.51	adrenergic, beta-2-, receptor, surface	−3.07
fibroblast growth factor receptor 3	2.48	GDF-1 embryonic GF cosmid R33485 containing pNORF1	−3.00
Wee1 + (S. pombe) homolog	2.42	major histocompatibility complex, class II, DQ beta 1	−2.96
selectin E (endothelial adhesion molecule 1); ELAM; CD62E	2.39	jun activation domain binding protein	−2.94
mitogen induced nuclear orphan receptor (MINOR) mRNA	2.36	villin2; ezrin	−2.91
G protein-coupled receptor 37 (endothelin receptor type B-like)	2.34	phoshatase &tensin homolog (mutated in multiple advanced cancers)	−2.78
KIAA0019 gene product	2.31		
CCAAT/enhancer binding protein (C/EBP), delta	2.28	src kinase-associated phosphoprotein of 55 kDa	−2.77
TCR adaptor molecule cbl-b	2.26	8-oxoguanine DNA glycosylase; hMMH; hOGG1	−2.71
fas (TNFRSF6)-associated via death domain, MORT1	2.25	platelet-derived growth factor receptor, beta polypeptide	−2.64
		suppressor of Ty (S. cerevisiae) 4 homolog 1	−2.57
		transcription factor 7 (T-cell specific, HMG-box)	−2.49

Table 3. Summary of the physicochemical properties of QDs used in this study.

QD type and coating	Core/shell shape	Core/shell diameter (nm)	Hydrodynamic diameter (nm)	Expected surface charge
QD 565-carboxylic acid	Spherical	4.6	14	Negative
QD 565-PEG-amine	Spherical	4.6	15	Positive
QD 655-carboxylic acid	Ellipsoid	6 (minor axis), 12 (major axis)	18	Negative

4 : Perform thermal cycling. Step 5 : Analyze changes in gene expression. The PCR Array Data Analysis Web Portal automatically performs the following calculations and interpretation of the control wells upon including threshold cycle data from a real-time instrument (Superarray, Fredrick, MD, USA)[19,20] (Figure 2).

Human cDNA Microarray

The methodology of Human cDNA Microarray was based on the procedures by DeRisi *et al.*[23]. For cDNA Radiolabeling, at first 2 μg of total RNAs prepared from prepared from the QD-treated HaCaT cells was used for each sample. To synthesize ^{33}P-labeled cDNAs, quantified RNA were labeled in a reverse transcription reaction containing 8 μL of 5X first standard PCR buffer, 4 μL of 24-mer poly dT primer, 4 μL of dNTP excluding dCTP, 4 μL of 0.1 M Dithiothreitol, 1 μL of RNase inhibitor, 6 μL of 3,000 Ci/mmol a-^{33}P dCTP and DEPC treated water to a final volume of 20 μL. And than, 2 μL of Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase was then added and the samples were incubated for 30 min at 42°C, followed by the addition of 2 μL of M-MLV reverse transcriptase and another incubation for 30 min at 42°C. 2.5 μL of 0.5 M Ethylene-Diamine-Tetra-Acetic Acid and 5 μL 0.1 M NaOH were added in order and the samples were incubated at 65°C for 30 min to hydrolyze remaining RNA. Following the addition of 12.5 μL of 1M Tris HCl (pH 8.0), the samples were purified using purification columns (Bio-rad, CA, USA). After purification, 4 mL of hybridization buffer was added each sample and reacted with nylon membrane during 24 hr.

Hybridization and Scanning

cDNA microarray was pre-hybridized in hybridization buffer containing 4 mL Microhyb and 10 μL of 8 mg/mL poly dA. Both Human Cot 1 and poly dA were

Table 4. Up & down regulated genes of HaCaT cells treated with QD-655-COOH.

Gene name	Z-ratio	Gene name	Z-ratio
amyloid beta (A4) precursor protein (protease nexin-II)	7.75	Bruton agammaglobulinemia tyrosine kinase	−4.40
MAX protein; helix-loop-helix zipper protein (max)	7.59	ESTs, weakly similar to eukaryotic initiation factor 4A-I	−4.15
ESTs, highly similar to fibroblast growth factor 12	5.59	ESTs, moderately similar to zinc finger protein ZNF49	−3.96
synuclein, alpha (non A4 component of amyloid precursor)	4.97	singed (Drosophila)-like (sea urchin fascin homolog like)	−3.69
MAD (mothers against decapentaplegic, Drosophila) homolog 5; Smad5	4.73	thyroid stimulating hormone receptor	−3.39
retinoblastoma-like 1 (p107)	4.57	ESTs, highly similar to EphB3	−3.32
cyclin B1	4.41	proteasome (prosome, macropain) subunit, alpha type, 1	−3.30
ESTs	4.26	interleukin 2 receptor, alpha	−3.25
no match on BLAST search	4.04	lymphocyte antigen 64 (mouse) homolog, radioprotective, 105 kD	−3.21
small inducible cytokine subfamily B (Cys-X-Cys), member 14 (BRAK)	3.74	suppressor of Ty (S. cerevisiae) 4 homolog 1	−3.13
tailless homolog (Drosophila)	3.66	adrenergic, beta-2-, receptor, surface	−3.10
selectin E (endothelial adhesion molecule 1); ELAM; CD62E	3.47	signal transducer and activator of transcription 2, 113 kD	−3.08
fibroblast growth factor receptor 3	3.32	chemokine (C-C motif) receptor 5	−3.00
mitoge induced nuclear orphan receptor (MINOR) mRNA	3.27	jun activation domain binding protein	−2.93
claudin 3	2.90	integrin, alpha 9	−2.68
Wee1+ (S. pombe) homolog	2.88	phoshatase &tensin homolog (mutated in multiple advanced cancers)	−2.54
breast cancer 1, early onset	2.83		
apoptosis-associated tyrosine kinase	2.79	GDF-1 embryonic GF; cosmid R33485 containing pNORF1	−2.54
Interleukin 9 receptor	2.65	8-oxoguanine DNA glycosylase; hMMH; hOGG1	−2.53
Interleukin 12B (natural killer cell stimulatory factor 2	2.61	ESTs, highly similar to NADH-ubiquinone oxidoreductase PDSW subunit	−2.39
retinol-binding protein 3, interstitial	2.60		
TCR adaptor molecule cbl-b	2.56	phosphogluconate dehydrogenase	−2.39
tumor necrosis factor receptor superfamily, member 1B; TRAIL receptor	2.50	lymphotoxin beta (TNF superfamily, member 3)	−2.35
zinc finger protein 162	2.29	DNA (cytosine-5-) methyltransferase 3 beta	−2.35
G protein-coupled receptor 37 (endothelin receptor type B-like)	2.28	Bcl-2-interacting killer (apoptosis-inducing)	−2.26
POU domain, class 2, associating factor 1	2.22	caspase 1, apoptosis-related cysteine protease	−2.26
platelet-activating factor receptor	2.13	mRNA for KIAA0553 protein, partial cds	−2.25
		villin2; ezrin	−2.23
		src kinase-associated phosphoprotein of 55 kDa	−2.23
		ubiquitin specific protease 8	−2.19

denatured at 95°C for 5 min before using. After 4 hr of pre-hybridization at 42°C, 10^7 cpm/mL of heat-denatured (95°C, 5 min) probe was added, and incubated at 42°C for 17 hr. Hybridized arrays were washed 3 times in 2X SCC and 0.1% SDS at room temperature for 15 min. The microarrays were exposed to phosphorimager screens for 1-5 days, and the screens were then scanned using a FLA-8000 (Fuji Photo Film Co., Japan) at 50 μm resolution[24,25].

Data Analysis

Microarray images were analzed using L-Processor (Fuji Photo Film Co) and the gene expression spots that produced by radioactive isotopes were counted by Arrayguage (Fuji Photo Film Co). Z scores provide each gene with the distance from the average intensity and were expressed in units of standard deviation. Gene expression difference as compared to untreated control cells was calculated by comparing the Z score differences among the same genes[24]. Z differences that calculated by subtracting Z scores of the control from each Z score of the samples were normalized again to distribute their position. These distributions represent the Z ratio value[26,27]. Cluster analysis was performed on Z-transformed microarray data by using two programs available as shareware from Michael Eisen's laboratory (http://rana.lbl.gov).

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