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Altered Gene Expression Profile After Exposure to Transforming Growth Factor β1 in the 253J Human Bladder Cancer Cell Line

Changho Lee1,2, Sang-Han Lee3, Doo Sang Kim1, Yun Soo Jeon1, Nam Kyu Lee1, Sang Eun Lee4

1Department of Urology, Soonchunhyang University Cheonan Hospital, Cheonan, 2Department of Urology, Seoul National University College of Medicine, Seoul, 3Department of Biochemistry, Soonchunhyang University College of Medicine, Cheonan, 4Department of Urology, Seoul National University Bundang Hospital, Seongnam, Korea

Purpose: Transforming growth factor β1 (TGF-β1) inhibits the growth of bladder cancer cells and this effect is prominent and constant in 253J bladder cancer cells. We performed a microarray analysis to search for genes that were altered after TGF-β1 treatment to understand the growth inhibitory action of TGF-β1.

Materials and Methods: 253J bladder cancer cells were exposed to TGF-β1 and total RNA was extracted at 6, 24, and 48 hours after exposure. The RNA was hybridized onto a human 22K oligonucleotide microarray and the data were analyzed by using GeneSpring 7.1.

Results: In the microarray analysis, a total of 1,974 genes showing changes of more than 2.0 fold were selected. The selected genes were further subdivided into five highly cohesive clusters with high probability according to the time-dependent expression pattern. A total of 310 genes showing changes of more than 2.0 fold in repeated arrays were identified by use of simple t-tests. Of these genes, those having a known function were listed according to clusters. Microarray analysis showed increased expression of molecules known to be related to Smad-dependent signal transduction, such as SARA and Smad4, and also those known to be related to the mitogen-activated protein kinase (MAPK) pathway, such as MAPKK1 and MAPKK4.

Conclusions: A list of genes showing significantly altered expression profiles after TGF-β1 treatment was made according to five highly cohesive clusters. The data suggest that the growth inhibitory effect of TGF-β1 in bladder cancer may occur through the Smad-dependent pathway, possibly via activation of the extracellular signal-related kinase 1 and Jun amino-terminal kinases Mitogen-activated protein kinase pathway.

Keywords: Cell line; Gene expression; Microarray analysis; Transforming growth factor beta; Urinary bladder neoplasms

INTRODUCTION

Transforming growth factor β (TGF-β) is a member of a family of dimeric polypeptide growth factors that includes bone morphogenic proteins and activins [1]. Every cell in the body, including epithelial, endothelial, hematopoietic, neuronal, and connective-tissue cells, produces TGF-β and has receptors for it [2]. Inhibition of cell proliferation is central to the TGF-β response in the epithelial lineage and escape from this response is a hallmark of many cancer cells [3].

In contrast with these opinions, we found that most bladder cancer cell lines are sensitive to the growth inhibitory action of TGF-β1 [4]. If human bladder cancers exhibit sensitivity to the growth inhibitory action of TGF-β1, TGF-β1 may be a strong candidate molecule for treating this hor-
rible disease.

In a previous study, we found that TGF-β1 inhibits the cellular growth of several bladder cancer cell lines. We thus assumed that bladder cancer cells are sensitive to the growth inhibitory action of TGF-β1. The aim of this study was to investigate how TGF-β1 inhibits the cellular growth of bladder cancer cells. For this, we investigated altered gene expression profiles after TGF-β1 treatment in 253J bladder cancer cells. We compared the altered gene expression profiles obtained with the currently suggested signal transduction pathway of TGF-β to infer the mechanism of the growth inhibition of 253J bladder cancer cells by TGF-β1.

MATERIALS AND METHODS

1. Cells and culture conditions

The human bladder cancer cell line 253J was obtained from the Korea Cell Line Bank (Seoul National University, Seoul, Korea). The cells were maintained in Dulbecco’s modified Eagle medium containing 10% fetal bovine serum, 100 units of penicillin/mL, and 100 µg of streptomycin/mL. TGF-β1 was purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2. RNA extraction

Total RNA was isolated from cells that were grown to approximately 60% confluence in 250-mL culture flasks (Sigma Chemical Co.) by use of TRI reagent (Gibco BRL/Life Technologies, Grand Island, NY, USA). The total RNA was phenol/chloroform-extracted, ethanol precipitated, and cleaned with RNeasy cleanup system columns (Qiagen, Valencia, CA, USA). The quantity and quality were determined by optical density measurements at 260 and 280 nm.

3. Microarray analysis

The human 22K oligonucleotide chip (Illumina Oligonucleotide Library, San Diego, CA, USA) was used in this study. Each 10 µg of total RNA was reverse transcribed in the presence of Cy3- or Cy5-dUTP (NEN Life Sciences, Boston, MA, USA) at 42°C for 2 hours. Control RNA was labeled with fluorescent Cy3-dUTP and test condition RNA was labeled with fluorescent Cy5-dUTP. Both the Cy3- and Cy5-labeled cDNA were purified by using the polymerase chain reaction (PCR) purification kit (Qiagen, Valencia, CA, USA) as recommended by the manufacturer. The purified cDNA was resuspended in 100 µL of hybridization solution containing 5× saline-sodium citrate (SSC), 0.1% sodium dodecyl sulfate (SDS), 30% formamide, 20 µg of Human Cot-1 DNA, 20 µg of poly A RNA, and 20 µg of yeast tRNA (Invitrogen, Grand Island, NY, USA). The hybridization mixtures were heated at 100°C for 2 to 3 minutes and were directly pipetted onto microarrays. The arrays were allowed to hybridize at 42°C for 12 to 16 hours in the humidified hybridization chamber (GenomicTree Inc., Daejeon, Korea). The hybridized microarrays were washed with 2× SSC/0.1% SDS for 5 minutes, 0.1× SSC/0.1% SDS for 10 minutes, and 0.1× SSC for 2 minutes two times. The washed microarrays were immediately dried by using the microarray centrifuge (GenomicTree Inc., Daejeon, Korea).

4. Microarray data acquisition

The hybridization images were analyzed by using GenePix Pro 4.0 (Axon Instruments, Union City, CA, USA). The average fluorescence intensity for each spot was calculated and local background was subtracted. All data normalization and selection of fold-changed genes were performed by using GeneSpring 7.1 (Silicon Genetics, Redwood City, CA, USA). The reliable genes were filtered with a cutoff value based on the two-component error model after intensity-dependent normalization (LOWESS) [5,6]. The averages of the normalized ratios were calculated by dividing the average of normalized signal channel intensity by the average of normalized control channel intensity. The analysis of variance test (parametric) and single t-test were performed at p-values of <0.01 or <0.05 to find genes that were differentially expressed across conditions. Unsupervised hierarchical clustering was performed by similarity measurements based on Pearson correlations around zero. Functional annotation of genes was performed according to the Gene Ontology Consortium (http://www.geneontology.org/index.shtml) by use of GeneSpring 7.1.

5. Expression of selected genes by RT-PCR

Total RNA from the Trizol isolate was treated with RNase-free DNase I. After removal of the DNase I, 1 µg of total RNA was reverse-transcribed by using random hexadeoxynucleotide primers. The genes of interest, Samd4, Gadd45β, TGF-β1, and the housekeeping gene β-actin, were analyzed by use of a Quantum RNA reverse transcription PCR (RT-PCR) kit according to the manufacturer’s protocol (Ambion, Austin, TX, USA). The following primers were used to amplify Samd4: forward, 5’-CCCGAGATCGTATGGTGGA-3’, and reverse, 5’-CCGGTCTCAAGTGGCAG-3’; Gadd45β: forward, 5’-GGAGGTTGGGGGCCTTC-3’, and reverse, 5’-GTGGAGGGTTCTGTCCG-3’ (451-bp); Smad3: forward, 5’-GGGCTCCCTATGCTCATCA-3’, and reverse, 5’-GGCTCGAGTGGATGACTTG-3’ (451-bp); TGF-β: forward, 5’-GCCCTGAAGACTTGTGATG-3’, and reverse, 5’-TCGCCCTCCCTGGTATAGGT-3’ (417-bp). β-actin primers (Ambion) were used as an internal standard (294 bp). PCR was performed as 94°C for 30 seconds, 56°C for 30 seconds, and 72°C for 30 seconds for 31 cycles, followed by a final elongation for 7 minutes. PCR products were electrophoresed on a 1.5% agarose gel and were visualized by ethidium bromide staining.

RESULTS

1. Gene expression patterns after TGF-β1 treatment in the 253J cell line

In the microarray analysis, the gene expression patterns

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FIG. 1. Hierarchical clustering of the gene expression profiles of 7,714 reliable genes (A) and 1,974 genes showing changes ≥2.0 fold in at least 1 array (B). Gene ontology map (C) and classification of 1,974 reliable genes according to GeneSpring 7.1 (D).

were observed at 6, 24, and 48 hours after TGF-β1 treatment. The genes were divided into TGF-β1-induced, TGF-β1-repressed, and other genes from 6 hours after the TGF-β1 treatment. The expression pattern was persistent and intensified for up to 48 hours after the TGF-β1 treatment. We filtered the analysis for reliable genes that had a sum of median >1,500 in 3 arrays (sum of median=Cy3 net signal+Cy5 net signal). This resulted in 7,714 genes.

Hierarchical cluster analysis was used to profile the gene expression patterns (Fig. 1A). Those genes showing changes of more than 2.0 fold in at least 1 array were selected. A total of 1,974 genes were found (Fig. 1B). These genes were classified by their known function (Fig. 1C, D). Because the expression difference between TGF-β treatment and the control was most remarkable at 48 hours after TGF-β treatment, we repeated the microarray at 48 hours. In this analysis, 7,992 reproducible genes were filtered, and a total of 310 genes showing similar expression in the two microarrays were selected by use of simple t-tests (Fig. 2). We then categorized these genes according to their known function (Table 1).

2. Cluster analysis
The genes showing changes of more than 2.0 fold in at least 1 array totaled 1,974 genes. Among them, a large number of genes were further subdivided into 5 highly cohesive clusters with high probability according to the time-dependent expression pattern (Fig. 3). After excluding genes whose name or function was not yet reported (i.e., hypothetical proteins), 270 genes were included in the cluster analysis. Other significant gene lists of 310 genes were obtained by using the two microarrays and simple t-tests (Fig. 2).

We selected 310 genes and performed a cluster analysis and made a gene list according to five different clusters. The gene profiles of each cluster are shown in Tables 2–6. Cluster 1 genes showed increased expression over the whole time period but were more up-regulated after 24
FIG. 2. Hierarchical clustering of the gene expression profiles of 7,992 reliable genes (A) and 310 statistically significant genes (B).

TABLE 1. Classification of 310 statistically significant genes as filtered by a repeat microarray

| Group | Description               | No. of genes |
|-------|---------------------------|--------------|
| 1     | Apoptosis regulator       | 8            |
| 2     | Cancer genes              | 3            |
| 3     | Cell cycle regulator      | 7            |
| 4     | Chaperone                 | 8            |
| 5     | Enzyme                    | 72           |
| 6     | Immunity protein          | 3            |
| 7     | Microtubular dynamics     | 1            |
| 8     | Motor                     | 1            |
| 9     | Nucleic acid binding      | 35           |
| 10    | Signal transducer         | 10           |
| 11    | Storage                   | 0            |
| 12    | Structural protein        | 32           |
| 13    | Transport                 | 34           |
| 14    | Unclassified              | 160          |

FIG. 3. Cluster analysis of the 310 statistically significant genes according to the time response. These genes were classified into 5 clusters by GeneMaths. Cluster 1 (red); showing increased expression over the whole time periods but more up-regulated after 24 hours sequentially. Cluster 2 (green); showing decreased expression over the whole time period. Cluster 3 (purple); without showing meaningful changes at 6 or 24 hours but becoming up-regulated at 48 hours, Cluster 4 (blue); without showing meaningful changes at 6 or 24 hours but becoming down-regulated at 48 hours, Cluster 5 (yellow); showing markedly increased expression over the whole time periods but becoming noticeably up-regulated only at 48 hours after transforming growth factor β1 treatment.

3. RT-PCR analysis

RT-PCR was used to evaluate a subset of genes identified by microarray analysis as undergoing significant changes in expression. We selected three interesting genes for RT-PCR confirmation: Samd4, Gadd45β, and TGF-β1. Specific primers were designed for the selected genes and mRNA expression was checked by RT-PCR with the use of β-actin as an internal standard. The expression of these genes in RT-PCR was comparable to the results obtained by microarray (Fig. 4).
Among 1,974 genes showing changes of more than 2.0 fold in at least one array, four representative Smad-dependent pathway-related genes were detected. These genes included TGIF, Smad4, Smad5, and SARA1. Microarray analysis showed increased expression of SARA and the common Smad, Smad4 (Fig. 5). This result indicated that the growth inhibitory signal transduction of TGF-β1 in bladder cancer might be mediated by the Smad-dependent pathway.

4. Assumption of the growth inhibitory pathway of TGF-β1 on 253J cells

1) Smad-dependent pathway
Among 1,974 genes showing changes of more than 2.0 fold in at least one array, four representative Smad-dependent pathway-related genes were detected. These genes included TGIF, Smad4, Smad5, and SARA1. Microarray analysis showed increased expression of SARA and the common Smad, Smad4 (Fig. 5). This result indicated that the growth inhibitory signal transduction of TGF-β1 in bladder cancer might be mediated by the Smad-dependent pathway.

2) Smad-independent pathway
Among the 1,974 genes, seven Smad-independent mitogen-activated protein kinase (MAPK) pathway-related genes were detected (Fig. 6). Of these, MAPKK1 and MAPKK4 expression were up-regulated. MAPKK1 is known to activate extracellular signal-related kinase 1 (ERK1), and MAPKK4 and MAPKK7 are known to activate Jun amino-terminal kinases (JNK). This result indicated that the growth inhibitory signal transduction of TGF-β1 in bladder cancer might be mediated by the Smad-independent MAPK pathway, especially the ERK1 and JNK pathways.
TABLE 3. Cluster 2: a total of 43 genes showed a tendency to decrease

| Gene name  | Gene bank accession no. | Protein description                             | Fold change  |
|------------|-------------------------|-------------------------------------------------|--------------|
|            |                         |                                                 | 6 h         |
|            |                         |                                                 | 24 h         |
|            |                         |                                                 | 48 h         |
| C4BPA      | NM_00715                | Complement component 4 binding protein, alpha   | 0.2         |
| GAN        | NM_022041               | Giant axonal neuropathy (gigaxonin)             | 0.9         |
| CASP8AP2   | NM_012115               | CASP8 associated protein 2                      | 0.2         |
| ZNF167     | NM_018651               | Zinc finger protein 167                        | 0.1         |
| NLGN4Y     | NM_014893               | Neuroligin 4, Y-linked                          | 0.4         |
| TUBGCP6    | NM_020461               | Tubulin, gamma complex associated protein 6     | 0.6         |
| SLMAP      | NM_007159               | Sarcolemma associated protein                   | 0.3         |
| RGPR       | NM_033127               | Regucalcin gene promotor region related protein | 0.7         |
| HOM-TES-103| NM_00731                | HOM-TES-103 tumor antigen-like                  | 0.6         |
| PRKCDBP    | NM_145040               | Protein kinase C, delta binding Protein          | 0.5         |
| SEC23A     | NM_006364               | Sec23 homolog A Saccharomyces cerevisiae         | 0.5         |
| ZNF43      | NM_003423               | Zinc finger protein 43 (HTP6)                   | 0.4         |
| SLC7A4     | NM_004173               | Solutecarrier family 7, member 4                | 0.6         |
| RNASE6     | NM_005615               | Ribonuclease, RNase A family, k6                | 1.0         |
| RUNX3      | NM_004350               | Runt-related transcription factor 3             | 0.6         |
| COL9A1     | NM_078485               | Collagen, type IX, alpha 1                      | 0.5         |
| CLMN       | NM_024734               | Calmin (calponin-like, transmembrane)           | 0.7         |
| ARHGEF1    | NM_004706               | Rho guanine nucleotide exchange factor (GEF) 1  | 0.6         |
| HYPE       | NM_007076               | Huntingtin interacting protein E                | 0.8         |
| ACOX1      | NM_007292               | Acyl-Coenzyme A oxidase 1, palmitoyl            | 0.1         |
| MMP25      | NM_004142               | Matrix metalloproteinase-like 1                 | 0.7         |
| RECSL1     | NM_005132               | RECS-like 1 (yeast)                             | 0.7         |
| MAL        | NM_002371               | Mal, T-cell differentiation Protein             | 0.8         |
| SE57-1     | NM_025214               | CTCL tumor antigen se57-1                      | 0.3         |
| CDKN2A     | NM_058196               | Cyclin-dependent kinase inhibitor 2A            | 0.7         |
| C22ORF1    | NM_001585               | Ch'some 22 open reading frame 1                 | 0.7         |
| SLC24A6    | NM_024959               | Solute carrier family 24, member 6              | 0.5         |
| SERPINA6   | NM_001756               | Serine proteinase inhibitor, clade A, member 6  | 0.5         |
| FOLR1      | NM_016730               | Folate receptor 1 (adult)                       | 0.7         |
| CRN7       | NM_024535               | Coronin 7                                       | 0.7         |
| EVER1      | NM_007267               | Epidermodysplasia verruciformis 1               | 0.7         |
| C9orf58    | NM_031426               | Chromosome 9 open reading frame 58              | 0.4         |
| MGLL       | NM_007283               | Monoglyceride lipase                            | 0.8         |
| LOC81558   | NM_030802               | C/EBP-induced protein                           | 0.5         |
| TAGLN      | NM_003186               | Transgelin                                      | 0.3         |
| KCNJ15     | NM_002243               | Potassium inwardly-rectifying channel, subfamily J, member 15 | 0.5       |
| RAD52      | NM_134422               | RAD52 homolog (S. cerevisiae)                   | 0.5         |
| TPP        | NM_007030               | Brain-specific protein p25 alpha                | 0.4         |
| AKIP       | NM_017900               | Aurora-A kinase interacting Protein             | 0.7         |
| KL1F       | NM_006563               | Kruppel-like factor 1 (erythroid)               | 0.1         |
| SLC30A5    | NM_024055               | Solute carrier family 30 (zinc transporter), member 5 | 0.6       |
| GN13       | NM_016541               | Guanine nucleotide binding protein (G protein), gamma 13 | 0.6     |
| HTR3B      | NM_006028               | 5-Hydroxytryptamine (serotonin) receptor 3B     | 0.8         |

TABLE 4. Cluster 3: a total of 3 genes showed no meaningful changes at 6 or 24 hours but significant up-regulation at 48 hours

| Gene name  | Gene bank accession no. | Protein description                             | Fold change  |
|------------|-------------------------|-------------------------------------------------|--------------|
|            |                         |                                                 | 6 h         |
|            |                         |                                                 | 24 h         |
|            |                         |                                                 | 48 h         |
| GOLPH4     | NM_014498               | Golgi phosphoprotein 4                          | 0.9         |
| RPHA       | NM_144563               | Ribose 5-phosphate isomerase A                  | 1.2         |
| PLK2       | NM_006622               | Polo-like kinase 2 (drosophila)                 | 1.2         |
The TGF-β treatment. A total of 7,714 altered genes were filtered after pressed, and other genes from 6 hours after the TGF-β genes were divided into TGF-β 48 hours after treatment. In the microarray analysis, the genes were classified along with their known function (Fig. 2A, B). We categorized these genes according to their expression in the two microarrays by use of simple t-tests microarray and found a total of 310 genes showing changes of more than 2.0 fold in at least one array.

### Table 5

| Gene name | Gene bank accession no. | Protein description | Fold change |
|-----------|-------------------------|---------------------|-------------|
| PCSK6     | NM_138319               | Proprotein convertase subtilisin/kexin type 6 | 1.5 0.7 0.4 |
| EGFR      | NM_005228               | Epidermal growth factor receptor | 0.8 0.9 0.3 |
| HCRTR1    | NM_001525               | Hyporeetin receptor 1 | 1.2 0.9 0.5 |
| PIK3R2    | NM_005027               | Phosphoinositide-3-kinase, regulatory subunit, polypeptide 2 | 0.8 1.2 0.5 |
| MMAB      | NM_052845               | Methylmalonic aciduria type B | 0.9 1.2 0.5 |
| CGI-14    | NM_015944               | CGI-14 protein | 1.2 1.2 0.5 |
| ABCF1     | NM_003090               | ATP-binding cassette, subfamily F (GCN20), member 1 | 1.0 1.1 0.5 |
| PRSS11    | NM_002775               | Protease, serine, 11 (IGF binding) | 1.0 0.8 0.4 |
| 8D6A      | NM_016579               | 8D6 antigen | 1.2 1.3 0.5 |
| MAGMAS    | NM_016089               | Mitochondria-associated protein involved in GMCSF signal transduction | 0.9 1.0 0.4 |
| CLN3      | NM_000086               | Ceroid-lipofuscinosis, neuronal3 | 0.9 1.0 0.5 |
| AKR1B1    | NM_001628               | Alde-keto reductase family 1, member B1 | 1.0 0.8 0.5 |

### Table 6

| Gene name | Gene bank accession no. | Protein description | Fold change |
|-----------|-------------------------|---------------------|-------------|
| PRE1      | NM_015387               | Preimplantation protein 3 | 1.9 1.9 2.4 |
| HEY1      | NM_012258               | Hair/split related with YRPW motif 1 | 1.4 2.7 3.0 |
| ZDHHC14   | NM_026030               | Zinc finger, DHH domain containing 14 | 1.9 1.4 2.0 |
| GC20      | NM_005875               | Translation factor sui1 homolog | 1.3 1.7 2.1 |
| GADD45B   | NM_015675               | Growth arrest and DNA-damage-inducible, beta | 2.8 3.4 6.8 |
| DGUOK     | NM_009177               | Deoxyguanosine kinase | 1.3 1.6 2.1 |
| RBPSUH    | NM_015874               | Recombining binding protein suppressor of hairless (Drosophila) | 1.4 1.5 2.1 |
| DDEF2     | NM_003887               | Development and differentiation enhancing factor 2 | 1.8 1.8 3.2 |
| CHPPR     | NM_014637               | Likely ortholog of chicken chondrocyte protein with a poly-proline region | 1.4 1.7 2.2 |
| ASE-1     | NM_012099               | CD3 epsilon-associated protein; antisense to ERCC-1 | 1.8 1.7 2.4 |
| ITGA6     | NM_002010               | Integrin, alpha 6 | 1.4 1.5 2.1 |
| ANGPTLA   | NM_139314               | Angiopoietin-like 4 | 5.1 7.8 6.8 |
| NUP153    | NM_005132               | Nucleoporin 153kDa | 1.5 1.5 2.3 |
| SYPL      | NM_006754               | Synaptophysin-like protein | 1.3 1.6 2.1 |
| NDRG1     | NM_006096               | N-myc downstream regulated gene 1 | 1.3 1.7 2.2 |
| ADA       | NM_000022               | Adenosine deaminase | 2.0 3.0 2.2 |
| PDLIM7    | NM_005451               | PDZ and LIM domain 7 | 1.8 3.0 2.9 |
| SCD       | NM_005063               | Stearoyl-CoA desaturase (delta-9-desaturase) | 1.5 2.7 4.6 |
| SLC12A1   | NM_000338               | Solute carrier family 12, member 1 | 2.7 3.4 6.3 |
| HMOX1     | NM_002133               | Heme oxygenase (decycling) 1 | 2.2 2.2 3.2 |
| SLC20A1   | NM_005415               | Solute carrier family 20, member 1 | 1.7 2.7 4.4 |

### Discussion

Microarray analysis was performed to search for genes that were altered after the TGF-β1 treatment to study the mechanisms of the growth inhibitory action of TGF-β1. 253J cells were selected because this cell line showed constant growth inhibition in the cell viability assays [4]. The 253J cells were treated with TGF-β1 and were harvested at 6, 24, and 48 hours after treatment. In the microarray analysis, the genes were divided into TGF-β1-induced, TGF-β1-repressed, and other genes from 6 hours after the TGF-β1 treatment. A total of 7,714 altered genes were filtered after the TGF-β1 treatment. Among these genes, the genes showing changes of more than 2.0 fold in at least one array were selected. A total of 1,974 genes were found. These genes were classified along with their known function (Fig. 1).

The microarray analysis was repeated by using 48-hour RNAs, because the expression pattern was persistent and intensified for up to 48 hours after the TGF-β1 treatment. We filtered 7,992 reproducible genes through the repeat microarray and found a total of 310 genes showing similar expression in the two microarrays by use of simple t-tests (Fig. 2A, B). We categorized these genes according to their known function. Seventy-two genes were categorized as genes associated with...
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Associated with nucleic acid binding, and another 34 genes were categorized as genes associated with transport. Thirty-two genes were classified in the structural protein category, 8 genes were associated with apoptosis, 3 genes were cancer genes, and 7 genes were associated with the cell cycle (Table 1).

We performed a cluster analysis to estimate the function of selected genes to gain insight into the mechanism of the TGF-β1 effect. Searching for meaningful information patterns in gene expression data is not trivial. An initial step was to cluster or group genes with similar changes in expression [7]. Moreover, we traced groups of genes showing similar expression patterns according to the time of expression and subdivided these into 5 highly cohesive clusters (Fig. 3). The cellular reaction process related to external stimuli or an altered internal milieu is generally sequential, and a variety of genes or proteins respond simultaneously. Thus, the assumption that genes showing a similar expression pattern according to reaction time will have a similar function may be true. According to this assumption, these selected 1,974 genes were further subdivided into 5 highly cohesive clusters with high probability according to the time-dependent expression pattern (Fig. 3).

We also made a list of the gene profiles of each cluster from a total of 310 genes showing similar expression in two microarrays by simple t-test analysis (Tables 1-5). On the basis of these two different gene lists, we could extract genes that not only belonged to the 310 genes but also were traced in the cluster analysis. This is the usual method used in microarray analysis to narrow a list of genes down to

| A | Smad4 | Smad4 | Smad4 | Smad4 | Smad4 |
|---|-------|-------|-------|-------|-------|
| -6 h | -24 h | -48 h | 5β-6 h | TGF-β1-6 h |
| Expression profile | 0.85 | 1.40 | 1.55 | 2.20 | 1.43 |

| B | Smad4 | Smad4 | Smad4 | Smad4 | Smad4 |
|---|-------|-------|-------|-------|-------|
| -6 h | -24 h | -48 h | 5β-6 h | TGF-β1-6 h |
| Fold change | 1.57 | 1.52 | 2.16 | 2.80 | 1.75 |

**Fig. 4.** Comparison expression profile on micrarray with the fold change on reverse transcription-polymerase chain reaction of the interesting genes.

**Fig. 5.** The expression patterns of the Smad-dependent pathway related genes. Most signal transducers showed elevated expression.

**Fig. 6.** The expression patterns of the MAP kinsae pathway related genes.
more significant genes. This gene list of 5 different clusters will be useful for elucidation of the TGF-β-mediated molecular pathways in mammalian cells.

Microarray data are often criticized owing to poor reproducibility [8]. To address this problem, we selected 3 interesting genes, such as Smad4, Gadd45β, and TGF-β1, and compared the microarray data with RT-PCR data. We can place some trust in the microarray data because the RT-PCR results were similar (Fig. 4).

To predict the mechanism by which TGF-β1 induces growth inhibition, we examined the altered gene expression profile obtained after TGF-β1 treatment on the basis of known TGF-β signal transduction pathways: the Smad-dependent pathway and the Smad-independent pathway [9,10]. We compared our microarray analysis results with known or suggested TGF-β signal pathways. We found some interesting signal molecules in our microarray data. The microarray analysis showed increased expression of SARA and the common Smad, Smad4. All of these genes are signal molecules regarded as part of the Smad-dependent TGF-β signal transduction pathway [10,11]. From these results, we can assume that the growth inhibitory signal transduction of TGF-β1 in bladder cancer might be mediated by the Smad-dependent pathway (Fig. 5). Moreover, the signal intensity of these molecules is intensified at 48 hours. This result correlated with the cellular response data in 253J cells after TGF-β1 treatment. 253J cells showed marked growth inhibition after TGF-β1 treatment and this inhibitory response was pronounced at 48 hours after treatment. We can assume that the pathway is more active at 48 hours after treatment.

Besides Smad-dependent signal transduction, we found 7 genes associated with the MAPK pathway. Among these, we found increased expression of MAPKK1 and MAPKK4. These two genes are critical to activating two different MAPK pathways, the ERK-1/2 pathway and the JNK1/2/3 pathway [12,13]. In the MAPK signal pathway, MAPKK1 is known to activate Erk1 and MAPKK4 is known to activate JUN [13,14]. Hence, we can assume that the mechanism of the growth inhibitory activity of TGF-β1 in bladder cancer cells may be through activation of the Erk1 and JNK MAPK signal pathways.

This work had some limitations. First, the gene list of altered expression was established from only one bladder cancer cell line, 253J. By comparing these data with results obtained from another appropriate bladder cancer cell line, such as T24 or 5637 cells, we might be able to narrow down the gene list. Secondly, the data from the microarray should be further confirmed by immunohistochemical staining or Western blotting. Nevertheless, our data have revealed several TGF-β1-inducible molecular pathways involving Smad4 and Gadd45β. Further characterization of these molecular pathways may elucidate the growth-inhibitory mechanism of TGF-β1 in bladder cancer.

CONCLUSIONS

A gene list showing significantly altered expression profiles after TGF-β1 treatment was developed according to five highly cohesive clusters. The data suggested that the growth inhibitory effect of TGF-β1 in bladder cancer may occur through the Smad-dependent pathway, possibly via activation of the Erk1 and JNK MAPK kinase pathways.

CONFLICTS OF INTEREST

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