KJU
Korean Journal of Urology

www.kjurology.org
http://dx.doi.org/10.4111/kju.2014.55.8.542

Original Article - Basic and Translational Research

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

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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*

Article History:
received 13 March, 2014
accepted 9 May, 2014

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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 *Smad4*: forward, 5'-CCCAGGATCAGTAGGTGGAA-3', and reverse, 5'-CCATGCCTGACAAGTTCTGA (451-bp); *Gadd45β*: forward, 5'-GGAAGGTTTTGGGCTCTCTG-3', and reverse, 5'-GTGTGAGGGTTCGTGACCAG-3' (471-bp); *Smad3*: forward, 5'-GGGCTCCCTCATGTCATCTA-3', and reverse, 5'-GGCTCGCAGTAGGTAACTGG-3' (441-bp); *TGF-β*: forward, 5'-GCCCTGAGAGACCTGCTGAA-3', and reverse, 5'-TCGCCTTCCCGTTGATAGTG-3' (147-bp). Beta-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-β treatment in the 253J cell line

In the microarray analysis, the gene expression patterns

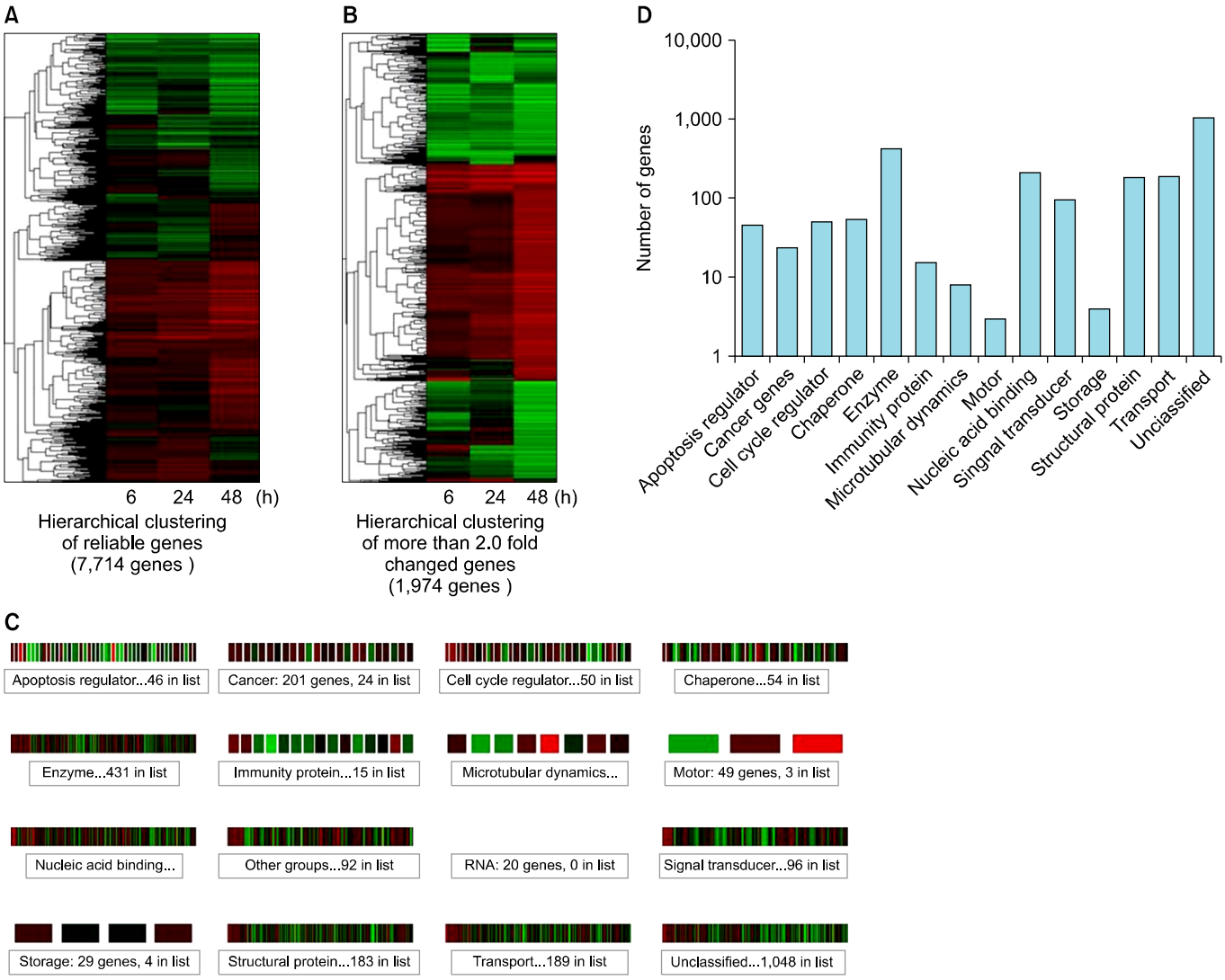

FIG. 1. Hierarchical clustering of the gene expression profiles of 7,714 reliable genes (A) and 1,974 genes showing changes ≥2.0 fold in at least 1 array (B). Gene ontology map (C) and classification of 1,974 reliable genes according to GeneSpring 7.1 (D).

were observed at 6, 24, and 48 hours after TGF-β 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

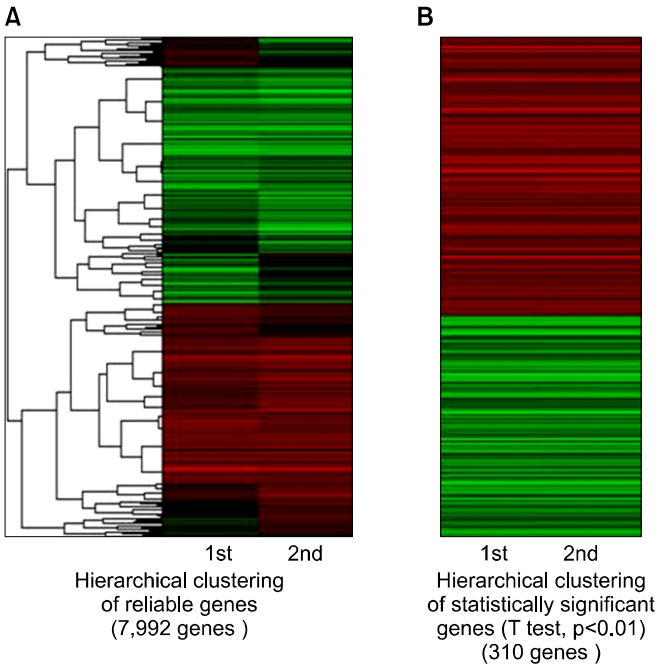

FIG. 2. Hierarchical clustering of the gene expression profiles of 7,992 reliable genes (A) and 310 statistically significant genes (B).

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

hours sequentially. Cluster 2 genes showed decreased expression over the whole time period. Cluster 3 genes showed meaningful changes at 6 or 24 hours but became up-regulated at 48 hours. Cluster 4 genes did not show meaningful changes at 6 or 24 hours but were down-regulated at 48 hours. Cluster 5 genes showed markedly increased expression over the whole time period but were noticeably up-regulated at 48 hours after TGF-β1 treatment.

3. RT-PCR analysis

RT-PCR was used to evaluate a subset of genes identified by microarray analysis as undergoing significant changes

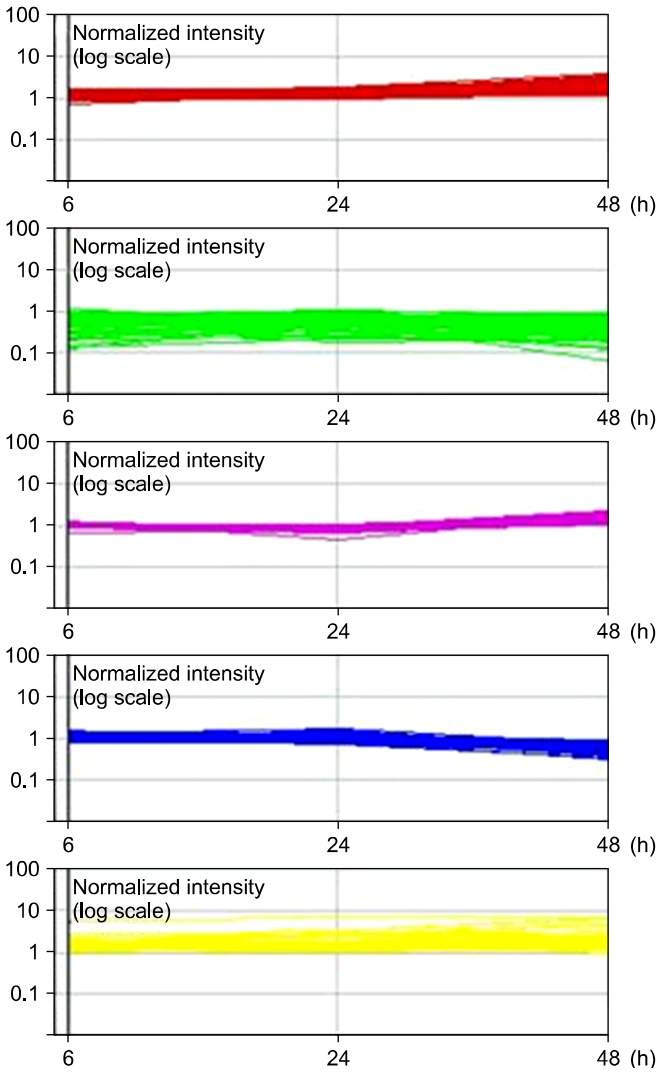

FIG. 3. Cluster analysis of the 310 statistically significant genes according to the time response. These genes were classified into 5 clusters by GeneMaths. Cluster 1 (red); showing increased expression over the whole time periods but more up-regulated after 24 hours sequentially, Cluster 2 (green); showing decreased expression over the whole time periods, 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.

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).

TABLE 2. Cluster 1: a total of 27 genes showed a steady increase in expression over the whole time range

Gene name	Gene bank accession no.	Protein description	Fold change		
			6 h	24 h	48 h
RAD54B	NM_006550	RAD54B	1.5	1.5	3.6
CBX1	NM_006807	Chromobox homolog 1	1.5	1.3	2.4
DER1	NM_018630	Derlin-1	1.4	1.4	2.5
RBBP4	NM_005610	Retinoblastoma binding protein 4	1.4	1.4	2.2
ITGB3BP	NM_014288	Integrin beta 3 binding Protein	1.3	1.2	2.2
RIF1	NM_018372	Receptor-interacting factor 1	1.2	1.4	2.2
CNOT7	NM_013354	CCR4-NOT transcription complex, subunit 7	1.5	1.4	2.4
PSMA2	NM_002787	Proteasome subunit, alpha type, 2	1.4	1.4	2.2
LEPRE1	NM_022356	Leucine proline-enriched Proteoglycan	1.1	1.3	2.3
GTF2H1	NM_005316	General transcription factor IIH	1.5	1.3	2.2
KIAA0934	XM_034536	catalytic activity	1.1	1.1	2.1
SEMA3C	NM_006379	Sema domain, immunoglobulin domain, short basic domain, secreted, (semaphorin) 3C	1.2	1.4	3.0
SUI1	NM_005801	Putative translation initiation factor	1.2	1.3	2.7
C13ORF7	NM_024546	Chromosome 13 open reading frame 7	1.2	1.1	2.4
ETFA	NM_000126	Electron-transfer-flavoprotein, alpha polypeptide	1.3	1.2	2.6
C12ORF14	NM_021238	Chromosome 12 open reading frame 14	1.5	1.6	3.0
EPHB4	NM_004444	EphB4	1.1	1.2	2.2
LOC54499	NM_019026	Putative membrane protein	1.4	1.4	2.5
PITPNB	NM_012399	Phosphatidylinositol transfer protein, beta	1.3	1.1	2.0
KIAA1078	XM_036589	Homo sapiens KIAA1078 Protein	1.7	1.6	3.5
COPA	NM_004371	Coatomer protein complex, subunit alpha	1.2	1.7	2.4
CD44	NM_000610	CD44 antigen, homing function and Indian blood group system	1.4	1.9	3.9
HECTD1	XM_030175	HECT domain containing 1	1.1	1.0	2.0
C21ORF7	NM_020152	Chromosome 21 open reading frame 7	1.2	1.4	2.3
UFM1	NM_016617	Ubiquitin-fold modifier 1	1.1	1.3	2.1
TULP4	NM_020245	Tubby like protein 4	1.7	1.6	3.4
SH3KBP1	NM_031892	SH3-domain kinase binding protein 1	1.2	1.3	2.0
MPZL1	NM_024569	Myelin protein zero-like 1	1.0	1.2	2.5
KIF21A	XM_040211	Kinesin family member 21A (KIF21A), mRNA.	1.3	1.1	2.2
APLP2	NM_001642	Amyloid beta (A4) precursor-like protein 2	1.2	1.4	2.8
SMBP	NM_020123	SM-11044 binding protein	1.1	1.2	2.2
PDCD4	NM_145341	Programmed cell death 4, neoplastic transformation inhibitor	1.1	1.5	2.2
NUDT4	NM_019094	Nucleoside diphosphate linked moiety X-type motif 4	1.1	1.1	2.4
FAT	NM_005245	FAT tumor suppressor homolog 1 (Drosophila)	1.4	1.3	2.2
LDOC1L	NM_032287	Leucine zipper, down-regulated in cancer 1-like	1.2	1.5	2.1
RAB10	NM_016131	Member RAS oncogene family	1.1	1.2	2.0
HNRPK	NM_002140	Heterogeneous nuclear ribonucleoprotein K	1.4	1.3	2.0

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 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 (ERK 1), and MAPKK4 and MAPKKK7 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 ERK 1 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_000715	Complement component 4 binding protein, alpha	0.2	0.4	0.5
GAN	NM_022041	Giant axonal neuropathy (gigaxonin)	0.9	0.6	0.4
CASP8AP2	NM_012115	CASP8 associated protein 2	0.2	0.2	0.3
ZNF167	NM_018651	Zinc finger protein 167	0.1	0.2	0.2
NLGN4Y	NM_014893	Neuroligin 4, Y-linked	0.4	0.3	0.2
TUBGCP6	NM_020461	Tubulin, gamma complex associated protein 6	0.6	0.6	0.4
SLMAP	NM_007159	Sarcolemma associated protein	0.3	0.2	0.2
RGPR	NM_033127	Regucalcin gene promotor region related protein	0.7	0.6	0.5
HOM-TES-103	NM_080731	HOM-TES-103 tumor antigen-like	0.6	0.5	0.6
PRKCDBP	NM_145040	Protein kinase C, delta binding Protein	0.5	0.7	0.7
SEC23A	NM_006364	Sec23 homolog A *Saccharomyces cerevisiae*	0.5	0.6	0.3
ZNF43	NM_003423	Zinc finger protein 43 (HTF6)	0.4	0.7	0.5
SLC7A4	NM_004173	Solutecarrier family 7, member 4	0.6	0.8	0.4
RNASE6	NM_005615	Ribonuclease, RNase A family, k6	1.0	0.5	0.6
RUNX3	NM_004350	Runt-related transcription factor 3	0.6	0.7	0.4
COL9A1	NM_078485	Collagen, type IX, alpha 1	0.5	0.7	0.3
CLMN	NM_024734	Calmin (calponin-like, transmembrane)	0.7	0.7	0.5
ARHGEF1	NM_004706	Rho guanine nucleotide exchange factor (GEF) 1	0.6	0.6	0.4
HYPE	NM_007076	Huntingtin interacting protein E	0.8	0.5	0.4
ACOX1	NM_007292	Acyl-Coenzyme A oxidase 1, palmitoyl	0.1	0.5	0.2
MMP25	NM_004142	Matrix metalloproteinase-like 1	0.7	0.5	0.4
REC8L1	NM_005132	REC8-like 1 (yeast)	0.7	0.9	0.3
MAL	NM_002371	Mal, T-cell differentiation Protein	0.8	0.7	0.3
SE57-1	NM_025214	CTCL tumor antigen se57-1	0.3	0.5	0.4
CDKN2A	NM_058196	Cyclin-dependent kinase inhibitor 2A	0.7	0.7	0.5
C22ORF1	NM_001585	Ch'some 22 open reading frame 1	0.7	0.8	0.3
SLC24A6	NM_024959	Solute carrier family 24, member 6	0.5	0.6	0.3
SERPINA6	NM_001756	Serine proteinase inhibitor, clade A, member 6	0.5	0.5	0.1
FOLR1	NM_016730	Folate receptor 1 (adult)	0.7	0.7	0.4
CRN7	NM_024535	Coronin 7	0.7	0.6	0.4
EVER1	NM_007267	Epidermodysplasia verruciformis 1	0.7	0.7	0.4
C9ORF58	NM_031426	Chromosome 9 open reading frame 58	0.4	0.4	0.1
MGLL	NM_007283	Monoglyceride lipase	0.8	0.5	0.4
LOC81558	NM_030802	C/EBP-induced protein	0.5	0.3	0.5
TAGLN	NM_003186	Transgelin	0.3	0.3	0.3
KCNJ15	NM_002243	Potassium inwardly-rectifying channel, subfamily J, member 15	0.5	0.6	0.7
RAD52	NM_134422	RAD52 homolog (*S. cerevisiae*)	0.5	0.4	0.4
TPPP	NM_007030	Brain-specific protein p25 alpha	0.4	0.4	0.4
AKIP	NM_017900	Aurora-A kinase interacting Protein	0.7	0.5	0.4
KLF1	NM_006563	Kruppel-like factor 1 (erythroid)	0.1	0.5	0.1
SLC30A5	NM_024055	Solute carrier family 30 (zinc transporter), member 5	0.6	0.8	0.5
GNG13	NM_016541	Guanine nucleotide binding protein (G protein), gamma 13	0.6	0.5	0.4
HTR3B	NM_006028	5-Hydroxytryptamine (serotonin) receptor 3B	0.8	0.3	0.7

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	0.9	2.0
RPIA	NM_144563	Ribose 5-phosphate isomerase A	1.2	0.8	2.0
PLK2	NM_006622	Polo-like kinase 2 (drosophila)	1.2	1.0	2.3

TABLE 5. Cluster 4: a total of 12 genes showed no meaningful changes at 6 or 24 hours but became down-regulated at 48 hours

Gene name	Gene bank accession no.	Protein description	Fold change		
			6 h	24 h	48 h
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	Hypocretin receptor 1	1.2	0.9	0.5
PIK3R2	NM_005027	Phosphoinositide-3-kinase, regu latory subunit, polypeptide 2	1.0	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_001090	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_016069	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	Aldo-keto reductase family 1, member B1	1.0	0.8	0.5

TABLE 6. Cluster 5: a total of 21 genes reacted with a noticeable increase in expression only at 48 hours

Gene name	Gene bank accession no.	Protein description	Fold change		
			6 h	24 h	48 h
PREI3	NM_015387	Preimplantation protein 3	1.9	1.9	2.4
HEY1	NM_012258	Hairy/enhancer-of-split related with YRPW motif 1	1.4	2.7	3.0
ZDHHC14	NM_024630	Zinc finger, DHHC 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_080917	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_000210	Integrin, alpha 6	1.4	1.5	2.1
ANGPTL4	NM_139314	Angiopoietin-like 4	5.1	7.8	6.8
NUP153	NM_005124	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 enzymes. Thirty-five genes were categorized as genes asso-

A

	Smad4 -6 h	Smad4 -24 h	Smad4 -48 h	Gadd4 5β-6 h	TGF- β1-6 h
Expression profile	0.85	1.40	1.55	2.20	1.43

B

	Smad4 -6 h	Smad4 -24 h	Smad4 -48 h	Gadd4 5β-6 h	TGF- β1-6 h
Fold change	1.57	1.52	2.16	2.80	1.75

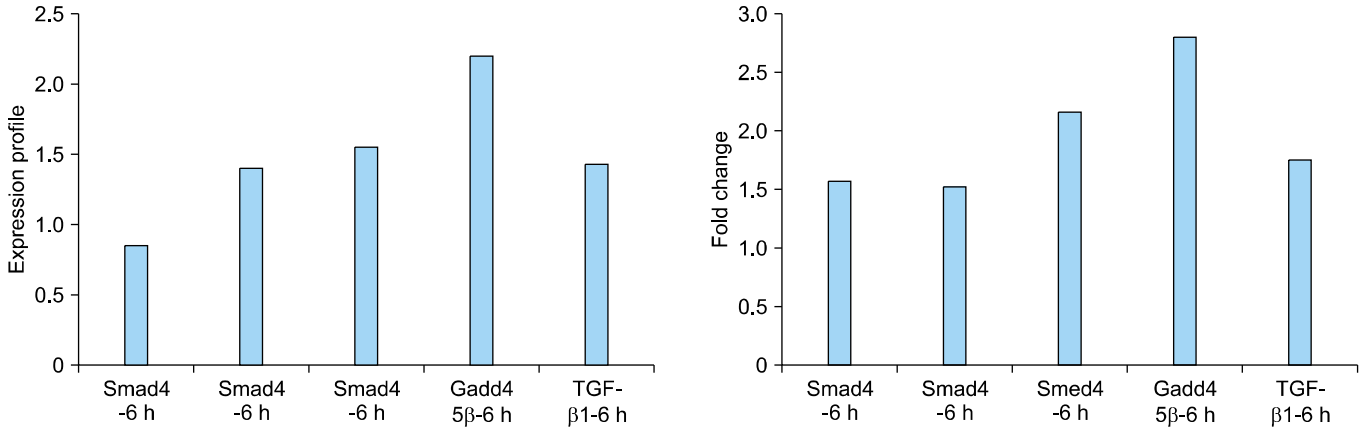

FIG. 4. Comparison expression profile on micrarray with the fold change on reverse transcription-polymerase chain reaction of the interesting genes.

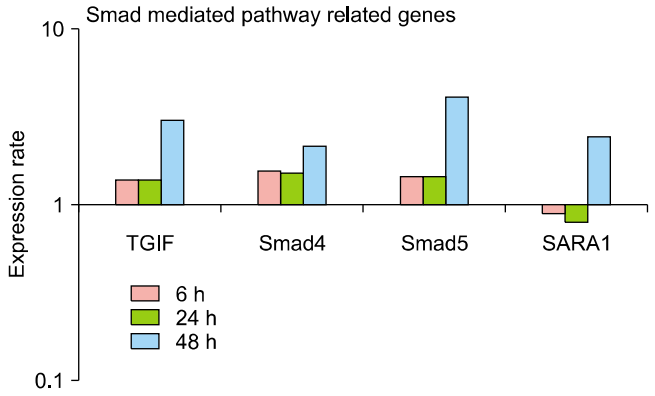

FIG. 5. The expression patterns of the Smad-dependent pathway related genes. Most signal transducers showed elevated expression.

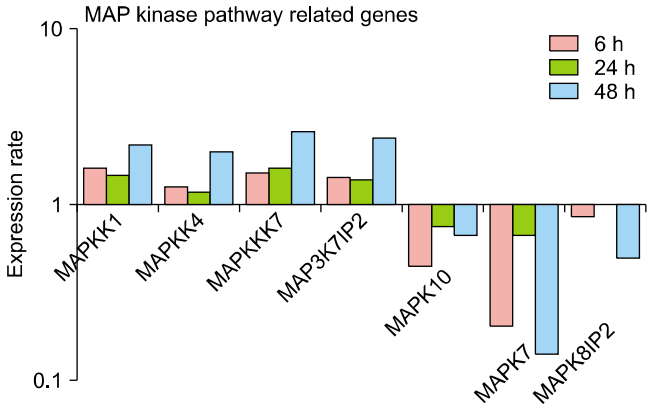

FIG. 6. The expression patterns of the MAP kinsae pathway related genes.

ciated 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

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

This research was supported, in part, by a grant from Sanofi-Aventis Korea Co., Ltd. The authors have nothing to disclose.

ACKNOWLEDGMENTS

This work received a research award from the Korean Urologic Association in 2008.

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