PDGF-BB를 처리한 hTERT 간성상세포주의 유전자 발현 양상과 Wnt 신호 활성 양상 분석

서울대학교 의과대학 병리학교실, 가톨릭대학교 의과대학 병리학교실¹

신혜원 • 박수영 • 이경분 • 신 은
남석우¹ • 이정용¹ • 장자준

Abstract

Transcriptional profiling and Wnt signaling activation in proliferation of human hepatic stellate cells induced by PDGF-BB

HyeWon Shin, M.S., Soo Young Park, M.S., Kyoung Bun Lee, M.D.,
Eun Shin, M.D., Suk Woo Nam, Ph.D.¹, Jung Young Lee, M.D.¹, Ja-June Jang, M.D.

Department of Pathology, Seoul National University College of Medicine;
¹The Catholic University of Korea College of Medicine, Seoul, Korea

Background/Aims: This study aimed to better understand gene expression profiles of human hepatic stellate cell (HSC) activation and the relationship with the Wnt signaling pathway. **Methods:** The global transcript levels in platelet derived growth factor-BB (PDGF-BB)-stimulated hTERT HSCs were analyzed using oligonucleotide microarrays. Oligonucleotide microarrays with 19K human oligo chips were performed to obtain gene expression profiles associated with proliferation in human hTERT HSCs. The microarray data was verified by real time quantitative PCR and expression of the components of Wnt signaling was analyzed by Western blot. **Results:** Microarray data showed 243 up-regulated and 265 down-regulated genes in PDGF-BB-treated HSCs. The changes in expression of glypican3 and BH3 interacting domain death agonist (BID) mRNA in real time quantitative PCR, especially among the highly up- or down-regulated genes, were statistically consistent with the microarray data. The Wnt signaling pathway components, frizzled10 (FZD10) and calcium/calmodulin-dependent protein kinase II alpha (CAMK2A), showed increased expression in the short time course microarray and the up-regulation of FZD10 also occurred at the protein level. Our data showed various gene expression profiles during activation of human HSC. **Conclusions:** The up-regulated expression of FZD10 and CAMK2A suggests that the Wnt/Ca²⁺ signaling pathway is active in hTERT HSCs and may participate in HSC activation and proliferation. (**Korean J Hepatol 2009;15:486-495**)

Key words: Hepatic stellate cells; PDGF-BB; Proliferation; Oligonucleotide microarray; Wnt signaling

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◇ Abbreviations: BID, BH3 interacting domain death agonist; CAMK2A, calcium/calmodulin-dependent protein kinase II alpha; Cy-3, cyanine-3; Cy-5, cyanine-5; DMEM, Dulbecco's minimal essential medium; FZD10, frizzled 10; GPC3, glypican 3; HSC, hepatic stellate cell; hTERT, human telomerase reverse transcriptase; PDGF, platelet derived growth factor

◇ Corresponding author: Ja-June Jang, E-mail: tripj@snu.ac.kr; Phone: 02) 740-8271; Fax: 02) 3673-5046

주소: 서울시 종로구 연건동 28, 서울대학교 의과대학 병리학교실 (우)110-799

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Introduction

Liver fibrosis is a common consequence of chronic liver injury regardless of its nature. Hepatic stellate cells (HSCs) are the major source of the constituent cells that play multiple roles in liver pathophysiology.¹ During liver fibrogenesis, HSCs undergo a process of activation by acquiring a myofibroblast-like phenotype that is characterized by increased proliferation, chemotaxis, and collagen synthesis.² Collagen-expressing activated HSCs have been shown to accumulate in patients with diseased livers.³

Normal human cells when in culture divide a limited number of times before entering a non-dividing state of replicative senescence. The telomeres undergo progressive shortening with successive cell divisions in a normal cell, and cellular senescence is speculated to be induced when the telomeres shorten to a critical length.⁴ Telomeres in human cells consist of a repetitive DNA sequence (TTAGGG)_n at the chromosome ends.⁵ This telomeric DNA is synthesized and maintained by telomerase, a ribonucleoprotein complex.⁶ A recent *in vitro* study has shown that the introduction and expression of human telomerase reverse transcriptase (hTERT) maintains telomere length and extends the life span of human fibroblasts.⁷ Accordingly, a human HSC immortalized with hTERT⁸ was selected for use in the present study.

The degree of fibrogenesis that occurs in liver diseases is most likely affected by the proliferation of HSCs.⁹ Results of *in vitro* and *in vivo* studies suggest that platelet derived growth factor (PDGF) is the most potent mitogen for HSCs, and is therefore likely to be an important mediator of increased HSC proliferation during hepatic fibrogenesis in chronic liver disease.¹⁰

The PDGF molecule is a dimer consisting of two peptide chains, referred to as the A- and B-chains. Of the three possible dimeric forms, (AA, AB, and BB), PDGF-BB has been shown to be the most effective stimulator of HSC proliferation.¹¹

Wnt signaling and their protein products have been shown to participate in the regulation of cellular differentiation, proliferation, and polarity.¹² Also, Wnt activity is enhanced in liver cirrhosis.¹² The Wnt signaling pathway consists of at least three downstream pathways: 1) the canonical pathway, 2) the planar cell polarity (PCP) pathway, and 3) the Wnt/Ca²⁺ pathway.¹³

In this study, to better understand the gene expression profile of human HSC activation at the molecular level, and to show the relationship with the Wnt signaling pathway, the global transcript levels in PDGF-BB-stimulated proliferating hTERT HSCs were analyzed using high-density spotted oligonucleotide microarrays.

Materials and Methods

1. Cell culture

Human hTERT immortalized HSCs were kindly given by Dr. KS Lee (Yonsei Medical University, Seoul, Korea). The cells were maintained on plastic tissue-culture flasks in Dulbecco's minimal essential medium (DMEM) with 10% fetal bovine serum supplemented with 1% penicillin and streptomycin and incubated at 37°C and 5% CO₂. In the experimental studies, the cells were plated on plastic tissue-culture dishes and grown for 1 day and then treated with 10 ng/mL of PDGF-BB (Upstate Biotechnology Inc., Lake Placid, NY, USA) in serum-free DMEM with 1% penicillin and streptomycin and 1% bovine serum albumin followed by

serum starvation with serum-free DMEM for 24 hours. Experimental treatments were conducted for 2, 4, 8, 12, 24, and 48 hours. All cells were incubated for 48 hours, and the cells of the 2–48 hour treatments were incubated in dilution buffer of PDGF-BB during the remaining study intervals. Cells used as controls were incubated in the dilution buffer for 48 hours.

2. Oligonucleotide microarrays

Total RNA was extracted from the PDGF-BB-treated cells by using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacture's protocols. The RNA quality was checked using RNA 6000 Nanochips on an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). High-density spotted oligonucleotide microarrays were manufactured at the array core facility of the Microdissection Genomics Research Center of Catholic University (Seoul, Korea). The Human OligoLibrary (Compugen/Sigma Genosys, Woodland, TX, USA) of 18,861 oligos was used in the 19K Human Oligoarray chip. A human universal reference RNA (Stratagene, La Jolla, CA, USA) was used as a reference RNA. A total RNA of 30 μ g was used for the oligonucleotide microarray. The reference RNA was labeled with cyanine-3 (Cy-3) and the test samples were labeled with cyanine-5 (Cy-5). Complementary DNA targets were reverse-transcribed from the total RNA using SuperScript II enzyme (Invitrogen, Carlsbad, CA, USA). The residual dye was removed by a Microcon YM-30 column (Millipore, Billerica, MA, USA). The Cy-5-labeled cDNA targets were hybridized with the Cy-3-labeled reference in a total of 40 μ L of hybridization solution [50% DIG EasyHyb (Roche, Basel, Switzerland) and 30 μ g of herring

sperm DNA (Promega, Madison, WI, USA)] for 18 hours at 42°C in a humidified conventional hybridization chamber (BioMicro Systems, Salt Lake City, UT, USA). After hybridization, the slides were washed with serial dilutions of 2X SSC, 0.1% SDS, 1X SSC, 0.2X SSC, and 0.05X SSC at room temperature for 2 minutes per wash step. The washed slides were scanned by a GenePix 4000B Scanner (Axon Instruments, Union City, CA, USA), and the Cy3/Cy5 signals were measured by GenePix Pro 3.0 microarray analysis software (Axon Instruments).

3. Real time quantitative PCR

Glypican 3 (GPC3) and BH3 interacting domain death agonist (BID), the two randomly selected genes in each highly up-regulated and down-regulated gene group were selected to verify the microarray data. From the total RNA of the PDGF-BB-treated cells extracted using TRIzol, cDNA was synthesized from 3 μ g of total RNA by reverse transcription (2 hours at 37°C), using 200 units of M-MLV reverse transcriptase (Promega, Madison, WI, USA) in a 30 μ L reaction mixture containing 0.5 μ g/ μ L random primers (Promega), 2.5 mmol/L GeneAmp® dNTPs (Applied Biosystems, Foster City, CA, USA), and 40 units of RNasin® ribonuclease inhibitor (Promega). Real time PCR was carried out in 20 μ L of reaction solution that consisted of 10 μ L of SYBR Premix Ex Taq (Takara, Shiga, Japan) and 0.4 μ L of ROX Reference Dye II (Takara), and 10 picomoles of primers. Forty cycles were carried out in an ABI Prism 7000 SDS (Applied Biosystems, Foster City, CA, USA), following cycles of 95°C for 20 seconds, 95°C for 5 seconds, and 60°C for 31 seconds. β -actin was used as an invariant control. The primer sequences are listed in Table 1.

Table 1. Primers used for real time quantitative PCR

Gene	Primer sequence	Size of PCR fragment
Glypican 3 (GPC3)	Sense 5'-TGATGATGGTTAAACCCCTGTGG-3'	234 bp
	Antisense 5'-CAATAGTGGTGGTCAGCTTTTCCT-3'	
BH3 interacting domain death agonist (BID)	Sense 5'-ATGGACTGTTGAGGTCAACAAC-3'	587 bp
	Antisense 5'-CAGTCCATCCCATTTCTGGCT-3'	

4. Western blot analysis

Total proteins were prepared according to a standard procedure.¹⁴ The total protein concentration was estimated by the Bradford assay, with BSA as a standard. Western blots were done using 50 μ g of protein and anti-FZD10 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-CamKII antibody (Santa Cruz Biotechnology). Beta-actin was used as a loading control.

5. Statistical analysis

Results were analyzed by independent-samples *t*-test using SPSS 12.0 software (SPSS, Inc., Chicago, IL, USA). Statistically significant differences between groups were defined as *P*-values<0.05. Studies were performed for at least three independent time periods and the results expressed as the mean $\pm$ SEM.

Results

1. Global changes in up-regulated and down-regulated gene expression of PDGF-BB-treated hTERT HSCs

The PDGF-BB microarray data showed a total of 10,399 gene expressions after block-wise normalization (Figure 1). The genes were hierarchically clustered into two groups: 1) controls and 2) PDGF-BB-treated for 2-48 hours. Next, 1,291 genes were filtered out through two-fold changes versus controls, and finally 560 genes

Figure 1. Hierarchical clustering identifies PDGF-BB-treated gene expression versus controls. A total of 10,399 genes were clustered in block-wise normalization. The four identical controls of PDGF-BB non-treated groups are numbered as PDGF 0-1, 0-2, 0-3, and 0-4.

which showed over two-fold changes at 24 and 48 hours versus controls were filtered out. The 24 hour group, as the peak time point that reflected the PDGF-BB effect, was selected for

Table 2. Genes highly upregulated in PDGF-BB-treated hTERT HSCs for 24 hours

Gene description	Chromosome location	Gene ID	Fold change*
Protein biosynthesis			
RPL12: ribosomal protein L12	9q34	Y11159	5.36
RPL5: ribosomal protein L5	1p22.1	NM_000969	2.33
Development			
HOXB9: homeobox B9	17q21.3	AK026084	4.66
C7orf16: chromosome 7 open reading frame 16	7p15	NM_006658	3.08
Proliferation			
POU3F2: POU domain class3 transcription factor2	6q16	NM_005604	4.27
ARHGEF1: Rho guanine nucleotide exchange factor1	19q13.1	AL_137705	3.07
Morphogenesis			
GPC3: glypican 3	Xq26.1	NM_004484	3.68
Transcription			
LHX2: LIM homeobox 2	9q33-q34.1	NM_004789	3.46
RAD54B: RAD54 homolog B	8q21.3-q22	NM_012415	3.35

*Fold change vs. mean of controls.

Table 3. Genes highly down-regulated in PDGF-BB-treated hTERT HSCs for 24 hours

Gene description	Chromosome location	Gene ID	Fold change*
Inflammatory response			
CCL20: chemokine (C-C motif) ligand 20	2q33-q37	NM_004591	-9.09
CCL2: chemokine (C-C motif) ligand 2	17q11.2-q1	NM_002982	-6.12
Transcription			
ZNF208: zinc finger protein 208	19p12	D70833	-8.69
SOD2: superoxide dismutase 2, mitochondrial	6q25.3	D17152	-5.58
Regulation of cell growth			
IL8: interleukin 8	4q13-q21	M17017	-7.94
NOV: nephroblastoma overexpressed gene	8q24.1	NM_002514	-3.10
Immune response			
IL7R: interleukin 7 receptor	5p13	NM_002185	-3.32
Apoptosis			
BAX: BCL2-associated X protein	19q13.3-q1	U92978	-2.75
BID: BH3 interacting domain death agonist	22q11.1	NM_001196	-2.18

*Fold change vs. mean of controls.

further analysis, since the cells seemed difficult to stand the serum free condition for 48 hours. A total of 243 genes were up-regulated and 265 genes down-regulated in the 24 hour data set. The most highly up-regulated and down-

regulated genes are listed in Tables 2 and 3.

2. Verification of the DNA microarray results

To verify the microarray data, two randomly

Figure 2. Verification of the microarray data by real time qPCR analysis. Messenger RNA levels of GPC3 and BID, the up-regulated and down-regulated gene in 24 hour PDGF-BB-treated hTERT HSCs, respectively, were significantly increased or decreased according to real time qPCR analysis. The data of GPC3 and BID is independent to each other.

* $P=0.018$ (GPC3), $P=0.009$ (BID), vs. control.

selected genes from each highly up-regulated and down-regulated PDGF-BB-treated group were assessed by real time quantitative PCR. GPC3 and BID showed a 3.68-fold increased and a 2.18-fold decreased expression, respectively, in the 24 hour PDGF-BB-treated group compared to the PDGF-BB non-treated group in the microarray data, and these genes also showed statistically increased or decreased expression in the real time quantitative PCR data (Figure 2). These results suggest that the data obtained by oligonucleotide microarray analysis are reliable and may be interpreted with confidence.

3. Components of the Wnt signaling pathway in PDGF-BB-treated hTERT HSCs

It was recently shown that Wnt signaling is active in HSCs and that Wnt signaling participates in hepatic fibrosis by promoting HSC activation and survival¹⁵, so we searched the components of the Wnt signaling pathway in the whole microarray data and obtained 11 genes,

Figure 3. Time course microarray data of two Wnt signaling pathway components. FZD10 and CAMK2A showed generally increased expression in the 48 hours of PDGF-BB treatment, even there are some shifts during the whole time course.

which are listed in Table 4. From examining the microarray data over the whole time course, we focused on two genes, frizzled 10 (FZD10) and calcium/calmodulin-dependent protein kinase II alpha (CAMK2A), showing a general increase during the time course (Figure 3). To examine the further translational levels of these genes, we performed Western blot analysis with PDGF-BB-non-treated and PDGF-BB-treated hTERT HSC proteins for the maximum time (48 hours). As a result, FZD10 was significantly increased in the 48 hour PDGF-BB-treated HSCs compared to the controls (Figure 4A, 4B); however, CAMK2A did not have any significant shifts (Figure 4A, 4C).

Discussion

Since HSCs are the major source of extra-cellular matrix and play a major role in liver fibrogenesis, it is important to understand the molecular mechanisms of HSC activation and

Table 4. Genes related to the Wnt signaling pathway in PDGF-BB-treated hTERT HSCs for 24 hours

Gene symbol	Gene title	Fold change*
PRKCB1	protein kinase C, beta 1	2.32
PPP2R2C	protein phosphatase 2A, gamma isoform	2.20
FZD 10	frizzled homolog 10 (<i>Drosophila</i>)	1.85
CAMK2A	calcium/calmodulin-dependent protein kinase II alpha	1.72
APC	adenomatosis polyposis coli	1.32
LEF 1	lymphoid enhancer-binding factor 1	1.29
PRKACB	protein kinase, cAMP-dependent, catalytic, beta	1.27
FZD 3	frizzled homolog 3 (<i>Drosophila</i>)	1.18
FOSL 1	FOS-like antigen 1	1.17
NFATC 1	nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 1	-1.58
DKK 1	dickkopf homolog 1 (<i>Xenopus laevis</i>)	-2.63

*Fold change vs. mean of controls.

proliferation involved in liver fibrogenesis. In this study, gene profiling of hTERT HSCs treated with PDGF-BB was performed over short time courses.

Unsupervised hierarchical clustering identified PDGF-BB-treated gene expressions versus controls. Many of the highest up-regulated and down-regulated genes were involved in cell proliferation, cell development, cell cycle, apoptosis, and immune responses.

In the validation through real-time quantitative PCR, GPC3 and BID, examples of the highly up-regulated and down-regulated genes in PDGF-BB-treated hTERT HSCs, respectively, showed significant changes as been indicated by the microarray data. GPC3 is a heparan sulfate proteoglycan that is bound to the cell membranes by a glycosyl-phosphatidylinositol anchor, which is known to be expressed in most hepatocellular carcinomas.¹⁶ Also, genetic and functional studies performed with *Drosophila*, *Xenopus*, zebrafish, and mammals have shown that glypicans are required for the optimal activity of Wnts, Hedgehogs, and bone morphogenic proteins in their specific tissues.¹⁷

BID belongs to the Bcl-2 protein family, and is primarily involved in the regulation of apoptosis at the mitochondrial level.¹⁸ The BH3-only proteins, which involve BID, are activated in response to different apoptotic stimuli at transcriptional or post-transcriptional levels.¹⁹ These proteins relay the signals to multi-domain Bax or Bak at the site of mitochondria, which induce the release of pro-apoptotic factors and a number of other events, leading to caspase activation and ultimately to cell death.²⁰

Furthermore, we searched the components of the Wnt signaling pathway in the whole microarray data and found several of the components exhibited up-regulated expressions. Among the eleven genes of Wnt signaling pathway we found, FZD10 and CAMK2A showed generally increasing expression during the short time course microarray data. The other nine genes also showed increased or decreased pattern but there were more random shifts over the time course, so they were excluded from the further study. Since the short time course microarray was not replicated but processed only once, it was not able to confirm the significance of the

Figure 4. Western blot analysis of translational levels of FZD10 and CAMK2A in PDGF-BB non-treated and PDGF-BB-treated hTERT HSC proteins for 48 hours (A). FZD10 was significantly increased in the 48 hour PDGF-BB-treated HSCs compared to controls (* $P=0.007$ vs. control) (B); however, CAMK2A did not show any significant changes (C). Actin was used as an internal control.

gene expressions over the time course, and it might be complemented in future studies. To examine whether these increased transcriptional levels of FZD10 and CAMK2A are continued to their translational levels, western blot was performed, and only protein expression of FZD10 was significantly increased. Since the Wnt-Frizzled signaling pathways are known to be intimately associated with differentiation, cell fate, and early development, this observation may suggest that FZD10 is one of the factors in HSC activation and proliferation. However, CAMK2A mRNAs seemed less translated at the protein level. Additional experiments would be necessary to explain the Wnt pathways related with CAMK2A mRNAs and proteins. Regarding the relationship between the Wnt signaling pathway and HSC activation, one recent study

suggested that activated rat HSCs implicated Wnt signaling pathway activation by activating the non-canonical Wnt signaling pathway²¹, which consists of the planar cell polarity pathway and the Wnt/ Ca^{2+} pathway. On the other hand, the recent study of Myung et al.¹⁵ indicated that Wnt signaling enhances the activation and the survival of human HSCs by the canonical Wnt pathway. Although the specific results are different in these studies and our study, it seems that Wnt signaling does activate HSCs, but more detailed studies are needed.

In conclusion, the overall gene expression in PDGF-BB-treated hTERT HSCs showed expression profiles of various genes regulating proliferation of HSCs during activation. Also, our results demonstrate that Wnt/ Ca^{2+} signaling are

active in hTERT HSCs and that it may participate in HSC activation and proliferation. Having the originality in demonstrating short time course oligonucleotide microarray of activated human hepatic stellate cell line to investigate genes related to hepatic stellate cell activation, the data reported herein may lead to an improved understanding of the mechanisms that underlie early fibrogenesis of human HSCs and searching specific target genes, although it is clear that additional specific studies are required.

요 약

목적: 간 정상세포의 증식은 간 섬유화 과정에서 중요한 역할을 한다. Platelet-derived growth factor (PDGF)는 매우 강력한 간정상세포 증식 촉진 물질이다. 사람의 간정상세포의 활성화에 관여하는 유전자 발현 양상과 Wnt신호 활성화 과정을 이해하기 위하여 PDGF-BB로 증식이 유도된 인체 간정상세포에서 올리고뉴클레오티드 마이크로어레이를 이용한 유전자 분석을 시행하였다. **대상과 방법:** 본 연구에서는 Telomerase 역전사 효소가 첨가된 불사멸화된 사람의 간정상세포주 (hTERT HSC)가 사용되었다. PDGF-BB의 세포증식 촉진 효과는 WST-1 실험을 통해 확인하였다. PDGF-BB를 처리한 hTERT HSC의 전체적인 유전자 발현 정도를 보기 위해 1만 9천개의 사람 유전자가 포함된 칩을 사용하여 올리고뉴클레오티드 마이크로어레이를 수행하였다. 마이크로어레이 데이터는 실시간의 정량적 중합효소연쇄반응으로 검증하였다. Wnt 신호 활성화는 웨스턴블롯으로 확인하였다. **결과:** PDGF-BB는 hTERT HSC의 증식을 강력하게 유도하였다. 마이크로어레이 데이터에서 PDGF-BB를 처리한 hTERT HSC의 경우 243개 유전자의 발현이 증가하였고 265개 유전자의 발현이 감소하였다. 무보정의 수직적인 군 분류 결과 PDGF-BB 처리군과 대조군은 유의하게 분리되었다. 마이크로어레이 데이터에서 대조군 대비 차이

가 가장 큰 유전자들 중 glypican3(GPC3), BH3 interacting domain death agonist(BID) mRNA 실시간 PCR에서의 발현 양상은 마이크로어레이 데이터와 통계적으로 상응하였다. Wnt 신호 전달계 중 frizzled10 (FZD10), calcium/calmodulin-dependent protein kinase II alpha (CAMK2A)는 시간의 경과에 따라 증가하였고 FZD10 단백질 발현 증가는 웨스턴블롯에서도 확인되었다. **결론:** PDGF-BB로 활성화된 인체 간정상세포의 전반적인 유전자 발현 및 Wnt 신호 전달계의 활성 양상은 간정상세포 활성 조절 유전자들을 찾는 데 기여할 것으로 기대된다. 이런 결과들은 활성화된 간정상세포에 의한 초기 간섬유화의 기전을 이해하는 데에 중요한 단서를 제공해 줄 것이다.

색인단어: 간정상세포, PDGF-BB, 증식, 마이크로어레이, Wnt 신호전달

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