

K562

Genistein
Apoptosis

Radiation-induced

* . * . * . * . * . * . *

_____ : K562 PTK inhibitor (herbimycinA genistein) apoptosis

_____ : K562 2×10^5 cells/mL

6-MV X-Ray 10 Gy (Clinac 1800C, Varian, USA) 200 300 cGy/min

Herbimycin A (HMA, Calbiochem, UK) genistein (Calbiochem, UK) di-
methylsulfoxide (DMSO, Sigma, UK) 1 mM 10 mM 250 nM 25 μ M

dization 128 cbnes . DNA sequencing

, GenBank database probe HMA genistein cbne

mRNA Northern hybridization

_____ : homo sapiens Smad6 95% cbne . probe

, genistein HMA genistein mRNA HMA

_____ : Smad6 apoptosis , Smad6가

apoptosis , genistein apoptosis

가 apoptosis

: , , K562, PTK , Smad6

¹⁾ HMA

apoptosis , genistein
apoptosis .
apoptosis

apoptosis PTK apoptosis

가 K562 , subtractive hybridization

apoptosis

가 protein tyrosine PCR-select cDNA subtraction

kinase (PTK) , herbimycin

A (HMA) genistein apoptosis ,

1999

2001 3 5 2001 6 8

suppression PCR 가 , sub-

Te1:051)240-5380 Fax :051)254-5889 가 가 ,

E- mail: hys ke @ da unet.do nga .ac .kr

traction

2, 3)

1.

K562 (ATCC CCL 243) American type of culture collection, 10% fetal bovine serum (Hyclone, USA) penicillin (100 units/mL)/streptomycin (100 µg/mL) (Gibco BRL, USA) RPMI 1640 (Gibco BRL, USA), 37, 5% CO₂ 가 2 × 10⁵ cells/mL

24

6-MV X-Ray (Clinac 1800C, Varian, USA) 200-300 cGy/min 10 Gy herbimycin A (HMA, Calbiochem, UK) genistein (Calbiochem, UK) dimethylsulfoxide (DMSO, Sigma, UK) 1 mM 10 mM 250 nM 25 µM

2. Subtraction hybridization

PCR-Select cDNA Subtraction Kit (Clontech, USA)

1) Adaptor Primer

oligonucleotide

cDNA synthesis primer ; 5'-TTTGTACAAGCTT30N IN-3'

Adaptor 1;

5'-CTAATACGACTCACTATAGGGCTCGAGCGGCCGCCCGGCAGGT-3' and 3'-GGCCCGTCCA-5'

Adaptor 2R;

5'-CTAATACGACTCACTATAGGGCAGCGTGGTCGCGGCCGAGGT-3' and 3'-GCCGGTCCA-5'

PCR primer 1; 5'-CTAATACGACTCACTATAGGGC-3'

Nested PCR primer 1; 5'-TCGAGCGGCCGCCCGGGCAGGT-3'

Nested PCR primer 2R; 5'-AGCGTGGTCGCGGCCGAGGT-3'

2) cDNA

(1) RNA

Total RNA Ultraspec-II reagent (Biotec Lab, USA) 가 , RNATack Resin

RNA diethylpyrocarbonate (DEPC, Sigma, UK)가

total RNA

PolyTract mRNA Isolation system (Promega, USA)

mRNA , biotin-labelled oligo (dT) 20 probe

mRNA streptavidin magnetic particles

(2) 가 cDNA

driver

genistein

tester

24

2 µg

mRNA 10 µM cDNA synthesis primer 가 ,

MJ Research PAC-150 Mini Cycler 70 , 2

avian myeloblastosis virus (AMV)-Reverse Transcriptase

(RT) 42 90 , 20×second strand

enzyme cocktail 가 16 2

T4 DNA polymerase (6 units) 가 . 16 30

20 × EDTA/glycogen 가

phenol extraction

(2.5 µL)

1% agarose gel

Rsa I

, tester cDNA Adap-

tor 1 Adaptor 2R

T4 DNA ligase

16

3) Hybridization

tube 3 µg driver cDNA 가 , 100

ng Adaptor 1-ligated tester Adaptor 2R-ligated tester

hybridization buffer , 98 90

68 8 hybridization . 68

tube 1.5 µg driver cDNA hybri-

dization buffer , 98 , 90

driver cDNA hybridization tube 가

. 68 20 , 200 µL buffer

가 7 -20

4) Selective PCR amplification

1 PCR hybridization 10 µM PCR

primer 1 75 , 5 95 , 1 ; 62 , 1

; 72 , 2 , 30 cycles, 72 , 10 1 cycle .

PCR 10 PCR 10 µM nested

PCR primer 1 2R , 94 , 1 ; 68 , 1 ; 72 ,

2 , 20 cycles 72 , 10 1 cycle . PCR

Perkin-Elmer 2400 PCR machine (Perkin-Elmer, USA)

, 2% agarose gel

3. DNA

E. coli XL1-Blue MRF',
 PCR pGEM-T easy vector system (Promega, USA),
 T4 DNA ligase, 2×rapid T4 DNA ligase buffer
 1/10 50% PEG (polyethylene glycol) 8000
 4 16 ampicillin (10 µg/
 mL) LB (Luria-Bertani)

4. PCR-selected dot hybridization

PCR-Selected Differential Screening Kit (Clontech, USA)
 . DNA
 clones PCR
 , 93 , 30 가 95 , 10 ; 68 , 3 . 28
 cycles PCR 96-well plate
 0.6 N NaOH DNA
 Hybond N-Plus Membrane (Amersham, UK) Bio-Dot Micro-
 filtration Apparatus (Bio-Rad, USA) , well
 DNA 가 driver
 tester cDNA random primed DNA labelling
 probe membrane 68
 hybridization . X-ray film -80 24
 , Fuji FPM 1200
 tester cDNA clones .

5. DNA sequencing

clones plasmid DNA Wizard Plus SV Mini-
 preps DNA Purification System (Promega, USA)
 , 20% PEG 6000 2.5 M NaOH
 . primers DNA
 ALFred M13-40 primer
 5'-cyanine-CGCCAGGGTTTTCCAGTCACGAC-3'
 ALFred MB Reverse primer
 5'-cyanine-TTTCACACAGGAAACAGCTATGAC-3'
 ALP Express Auto-
 Cycle Sequencing Kit (Pharmacia Biotech, UK)
 , 95 , 2 가 , 95 , 40 ; 55 , 40
 ; 72 , 1 , 25 cycles, 72 , 10 PCR
 . 5.5% Hydrolinker Long Ranger gel (FMC, USA) loading
 , ALP Express AutoCycle Sequencing System (Pharmacia
 Biotech, UK) B

DNA EMBL (European Mole-
 cular Biology laboratory) GenBank databases

6. Northern hybridization

30 µg total RNA 1.5% agarose-formaldehyde gel
 Sambrook ¹⁴⁾ Hybond N-Plus
 Membrane (Amersham, UK) capillary
 , membrane Spectrolinker XL-1000 UV crosslinker
 (Spectronics, USA) . probe clone
 plasmid DNA , PCR DNA
 . 1.5% agarose gel Qiaex II
 Gel Extraction Kit (Qiagen, USA) , rediprime
 Random Primer Labelling Kit (Amersham, UK) ,
 RNA가 membrane hybridization
 . formamide (Sigma, UK) 50 mL mixed-bed resin
 (Bio-Rad, USA) 5 g 4 4 가
 deionized formamide hybridization buffer
 가 . membrane hybridization buffer 42
 2 prehybridization . buffer
 [-³²P] dCTP labelled-cDNA probe 95 5
 가 가 42 hybridization
 . membrane 42 2×SSC/0.1%
 SDS, 1×SSC/0.1% SDS, 0.1×SSC/0.1% SDS
 5 2 . X-ray film -80
 24 Fuji FPM 1200

1. Subtractive hybridization

'driver' genistein
 'tester' . Driver
 tester mRNA cDNA hy-
 bridization PCR
 PCR . T/A-vector cloning 218
 , nested PCR primer 1 2R
 . PCR dot blotting
 clone .
 2.
 clones DNA blast search
 , homo sapiens Smad6

* gb|AF041065.1|HSS MAD6S4

Homo sapiens Smad6 protein (SMAD6) gene, exon 4 and complete cds

- Length = 1677
- Score = 339 bits (168), Expect = 2e-91
- Identities = 196/206 (95%), Gaps = 2/206 (0%)
- Strand = Plus / Plus

Query : 1 gtagaccaggccgtcagcatctctacgacctactcaggcagcggcttctgcctggg 60
 Sbjct : 185 gtagaccaggccgtcagcatctctacgacctactcaggcagcggcttctgcctggg 244
 Query : 61 ccagctcaacctggggcagcgcasrvggcgggtgctggcgaacgscgagcaagatcg-ct 119
 Sbjct : 245 ccagctcaacctggagcagcgcagcagtcgg-tgcggcgaacgcgagcaagatcgct 303
 Query : 120 tbggcatctgctcagcaaggagcccsacggcgtgtggcctacaaccggcgagcacc 179
 Sbjct : 304 tcggcatctgctcagcaaggagcccgacggcgtgtggcctacaaccggcgagcacc 363
 Query : 180 ccatcttcgtcaactccccgacgctg 205
 Sbjct : 364 ccatcttcgtcaactccccgacgctg 389

Fig. 1. Sequences alignment of Smad6 gene. Query : differentially expressed clone in this study, Sbjct : homo sapiens Smad6 gene.

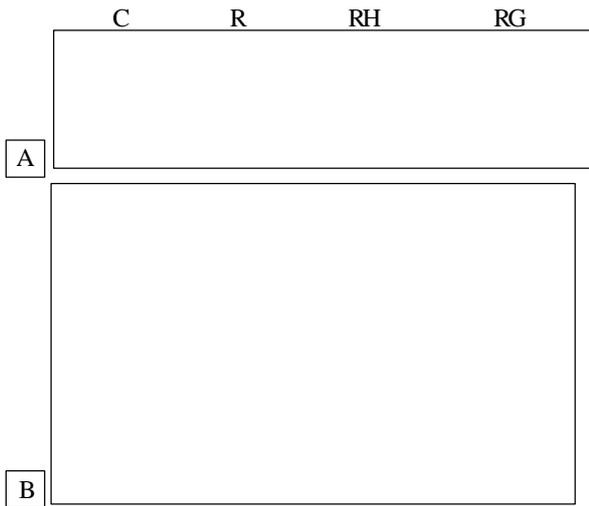
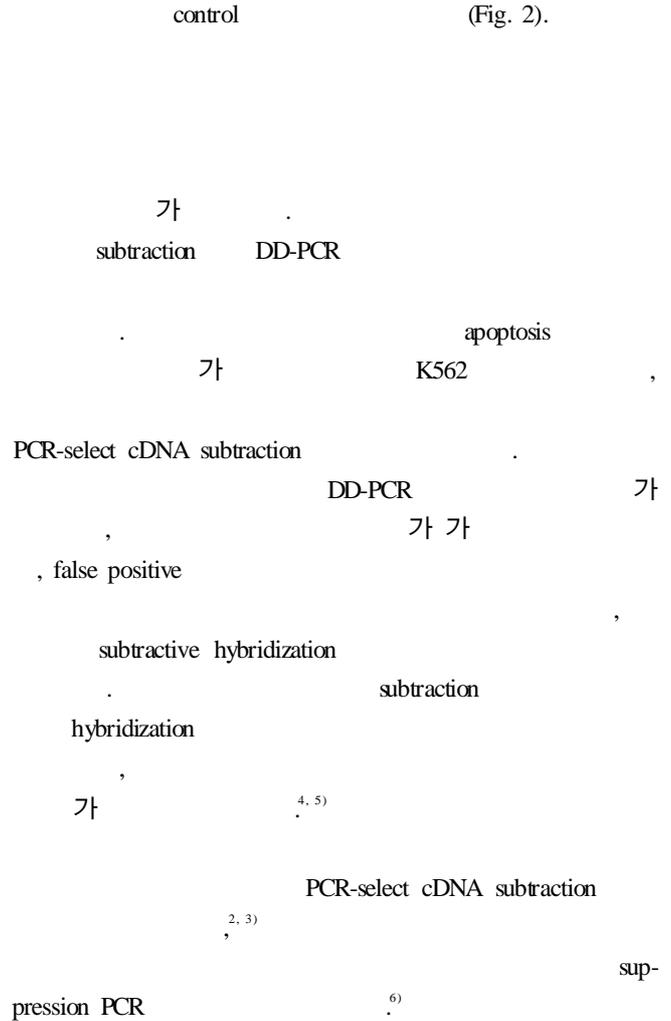


Fig. 2. Northern hybridization analysis of Smad6 gene. (A) Comparison of mRNA expression. Lane 1: untreated (C), Lane 2: 10 Gy X-ray irradiated (R; driver), Lane 3: combined treated with irradiation and 250 nM HMA (RH), Lane 4: combined treated with irradiation and 25 μM genistein (RG; tester). (B) Ethidium Bromide staining of 18S and 28S rRNA was used as a loading standard.



95%

clone

Fig. 1

3. Smad6 mRNA

genistein

HMA

HMA

pression PCR

genistein Smad6 apoptosis mouse hybridoma
 zation 가 subtractive hybridi- MH60 apoptosis BMP2
 genistein “tester” Smad6가 BMP2 TGF
 cDNA subtractive hybridization TAK1 p38 apoptosis
 clones DNA sequencing Smad1 Smad2 3 Erk/
 blast search MAPK . Erk
 homo sapiens Smad6 95% Smad1 Smad1
 clone , Northern hybridization mRNA BMP .²²⁾
 driver , tester cDNA Smad6
 HMA mouse B cell hybridoma HS-72 Smad6 hBMP-
 Smad6 가 induced growth arrest apoptosis ,
 가 Smad6가 B lineage hBMP2 growth arrest
 apoptosis negative feedback loop
 Smad6 Smad1 5
 Smad6 Smad family subfamily . BMP-induced signal growth arrest
 Smad family *Drosophila Mad* apoptosis .²³⁾ Smad6가
 Protein) , TGF- /BMP (Bone Morphogenetic apoptosis
 downstream effector .⁷⁾
 8 subfamily Smad6 mRNA dirver
 . Smad 45 70 kDa 가 tester 가 HMA
 N- C- mothers against decapentaplegic
 (mad) homology (MH 1 MH 2) .⁸⁾ Smad 가
 , TGF- mouse 가
⁹⁾ Smad6 Smad7 .²⁴⁾ IL-1, TNF, IL-12, SCF (stem cell factor)
 Smad “inhibitory Smad” cytokine
 TGF- .²⁵⁾ Smad6가 TGF-
 serine/threonine kinase receptors , type I effector Smad
 receptor ,¹⁰⁾ Smad4 Smad6
 Smad subfamily heteromeric complex apoptosis
 Smad subfamily .¹¹⁾ Smad6
 Smad7 mRNA shear stress ,
 .¹²⁾ Smad6 7 . Smad6
 , Smad7 TGF family antisense Smad6
 Smad6 BMP signal
^{11, 13, 15, 16)}
 Smad6 7 . Smad6
 mRNA .¹⁷⁾ SH2 Smad6 PTK
 family SOCS/JAB/SSI가 JAK/STAT signaling anta- Smad6 upstream
 gonist Smad6 .^{18 20)} 가

1. , , . K562 Apoptosis PTK Inhibitors 2000;18:51-58
2. Diatchenko L, Chenchik A Siebert P. Suppression subtractive hybridization: A method of generating differentially regulated or tissue-specific cDNA probes and libraries. *Proc Natl Acad Sci USA* 1996; 93:6025-6030
3. Gurskaya NG, Diatchenko L, Chenchik A et al. Equalizing cDNA subtraction based on selective suppression of polymerase chain reaction: Cloning of Jurkat cell transcripts induced by phytohemagglutinin and phorbol 12-myristate 13-acetate. *Anal Biochem* 1996; 240:90-97
4. Duguid JR and Dinauer MC. Library subtraction of in vitro cDNA libraries to identify differentially expressed genes in scrapie infection. *Nucl Acids Res* 1990;18:2789-2792
5. Hara E, Kato T, Nakada S, Sekiya S, Oda K. Subtractive cDNA cloning using oligo (dT)30-tex and PCR: Isolation of cDNA clones specific to undifferentiated human embryonal carcinoma cells. *Nucl Acids Res* 1991;19:7097-7104
6. Sieber PD, Chenchik A, Kellogg DE, Lukyanov KA. An improved method for walking in uncloned genomic DNA. *Nucl Acids Res* 1995;23:2598-2602
7. Massague J, Hata A, Liu F. TGF signalling through the Smad pathway. *Trends Cell Biol* 1997;7:187-192
8. Hehlh CH, Miyazono K, Dijke P. TGF-beta signalling from cell membrane to nucleus through Smad proteins. *Nature* 1997;390:465-471
9. Li M, Li J, Hoodless PA. Mothers against decapentaplegic-related protein 2 expression in avian granulosa cells is up-regulated by transforming growth factor beta during ovarian follicular development. *Endocrinology* 1997;138:3659-3665
10. Tsuneizumi K, Nakayama T, Kamoshida Y, Kornberg TB, Christian JL, Tabata T. Daughters against dpp modulates dpp organizing activity in *Drosophila* wing development. *Nature* 1997;389:627-631
11. Hata A, Lagna G, Massague J, Hemmati-Brivanlou A. Smad6 inhibits BMP/Smad1 signaling by specifically competing with the Smad4 tumor suppressor. *Genes Dev* 1998;12:186-197
12. Topper JN, Cai J, Qui Y et al. Vascular MADs: two novel MAD-related genes selectively inducible by flow in human vascular endothelium. *Proc Natl Acad Sci USA* 1997;94:9314-9319
13. Imamura T, Takase M, Nishihara A. Smad6 inhibits signalling by the TGF-beta superfamily. *Nature* 1997;389:622-626
14. Sambrook J and Russell DW. Molecular Cloning, Chapter 6. In: Preparation and analysis of eukaryotic genomic DNA, Argentine J, Third ed. New York, USA, 2001;6:39-646
15. Souchehytskyi S, Nakayama T, Nakao A et al. Physical and functional interaction of murine and *Xenopus* Smad7 with bone morphogenetic protein receptors and transforming growth factor-beta receptors. *J Biol Chem* 1998;273:25364-25370
16. Bai S, Shi X, Yang X, Cao X. Smad6 as a transcriptional corepressor. *J Biol Chem* 2000;275:8267-8270
17. Afrakhte M, Moren A, Jossan S et al. Induction of inhibitory Smad6 and Smad7 mRNA by TGF-beta family members. *Biochem Biophys Res Commun* 1998;249:505-511
18. Starr R, Wilson TA, Viney EM et al. A family of cytokine-inducible inhibitors of signalling. *Nature* 1997;387:917-921
19. Endo TA, Masuhara M, Yokouchi M et al. A new protein containing an SH2 domain that inhibits JAK kinases. *Nature* 1997;387:921-924
20. Naka T, Narazaki M, Hirata M et al. Structure and function of a new STAT-induced STAT inhibitor. *Nature* 1997;387:924-929
21. Kimura N, Matsuo R, Shibuya H, Nakashima K, Taga T. BMP2-induced apoptosis is mediated by activation of TASK1-p38 kinase pathway that is negatively regulated by Smad6. *J Biol Chem* 2000;275(23):17647-17652
22. Kretschmar M, Doody J, Massague J. Opposing BMP and EGF signalling pathways converge on the TGF family mediator Smad1. *Nature* 1997;389:618-622
23. Ishizaki A, Yamato K, Hashimoto S et al. Differential inhibition of Smad6 and Smad7 on bone morphogenetic protein- and activin-mediated growth arrest and apoptosis in B cells. *J Biol Chem* 1999;274:13637-13642
24. Vodovotz Y, Lucia MS, De Lucca AM, Mitchell JB, Kopp JB. Reduced hematopoietic function and enhanced radiosensitivity of transforming growth factor-beta1 transgenic mouse. *Int J Cancer* 2000;90:13-21
25. Neta R. Modulation with cytokines of radiation injury: Suggested mechanisms of action. *Environ Health Perspect* 1997;105 suppl.6:1463-1465

Abstract

**Smad6 Gene and Suppression of Radiation- Induced Apoptosis by
Genistein in K562 Cells**

Soo Jin Jeong, M.S.^{*}, Young Hee Jin, M.S.^{*}, Yeo Jin Yoo, M.D.^{*},
Chang Ho Do, M.D.^{*}, Min Ho Jeong, M.D.^{*}, Gi Yeong Huh, M.D.^{*},
Hye Ran Bae, M.D.^{*}, Kwang Mo Yang, M.D., Chang Woo Moon, M.D.,
Sin Geun Oh, M.D., Won Joo Hur, M.D. and Hyung Sik Lee, M.D.

Department of Radiation Oncology, Institute of Medical Science^{*},
College of Medicine, Dong- A University, Pusan, Korea

Purpose : The genes involved on the suppression of radiation-induced apoptosis by genistein in K562 leukemia cell line was investigated.

Materials and methods : K562 cells in exponential growth phase were irradiated with a linear accelerator at room temperature. For X-ray irradiation and drug treatment, cultures were prepared at 2×10^5 cells/mL. The cells were irradiated with 10 Gy (Clinac 1800C, Varian, USA). Stock solutions of herbimycin A (HMA, Calbiochem, UK) and genistein (Calbiochem, UK) were prepared in dimethylsulfoxide (DMSO, Sigma, UK). After incubation at 37 for 24 h, PCR-select cDNA subtractive hybridization, dot hybridization, DNA sequencing and Northern hybridization were examined.

Results : Smad6 gene was identified from the differentially expressed genes in K562 cells incubated with genistein which had been selected by PCR-select cDNA subtractive hybridization. The mRNA expression of Smad6 in K562 cells incubated with genistein was also higher than control group by Northern hybridization analysis.

Conclusion : We have shown that Smad6 involved on the suppression of radiation-induced apoptosis by genistein in K562 leukemia cell line. It is plausible that the relationship between Smad6 and the suppression of radiation-induced apoptosis is essential for treatment development based on molecular targeting designed to modify radiation-induced apoptosis.

Key Words : PTK inhibitors, Oncogene expression, Radiation-induced apoptosis, Smad6