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# Identification of Differentially Expressed Genes by Proto-oncogene Protein DEK using Annealing Control Primers

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**Abstract** – The proto-oncogene protein DEK has been implicated in various human disease including cancer. We have shown that DEK induces caspase-dependent apoptosis in *Drosophila* by regulating histone acetylation. Reverse transcription-polymerase chain reaction (RT-PCR) method based on annealing control primers was used to screen and identify differentially expressed genes (DEGs) in DEK overexpressed HeLa cells. Among the genes identified, clusterin and fibrillarin have major role in apoptosis pathway regulation. TFIIIC and RPS24 are implicated in HAT mediated transcriptional initiation and cololectal cancer, respectively. To further analyze DEK's role in apoptosis, multiplex PCR was performed. Caspase-3, -7, and -10 and proapoptotic gene bid were newly identified as possible target genes regulated by DEK expression.

**Keywords:** DEK, Apoptosis, Annealing control primer, Differentially expressed gene, GeneFishing

## INTRODUCTION

Initially identified in a fusion with the CAN nucleoporin protein, DEK is involved in acute myelogenous leukemia (AML) subtype with a translocation (6;9) which results in the formation of a DEK-CAN fusion gene (von Lindern *et al*., 1990). As a nuclear phosphorprotein, DEK possesses the major DNA binding domain (DBD) SAP box, and at least three highly acidic domains (Waldmann *et al*., 2004). The acidic domain harboring the N-terminal two-thirds of the DEK protein has been shown to be fused to the C-terminal two-thirds of CAN in the DEK-CAN fusion protein (von Lindern *et al*., 1992). We have identified that p300- and PCAF-mediated histone acetyltransferase (HAT) inhibitory activities of DEK through its acidic domain containing regions (Ko *et al*., 2006). Previous reports have suggested that the DEK proto-oncogene protein performs vital functions in chromatin remodeling, via the alteration of the topology of chromatin (Waldmann *et al*., 2002). It has been proposed that DEK is one component of a multiprotein transcriptional repressor complex which includes hDaxx and HDAC2 (Hollenbach *et al*., 2002). Recent report suggests that DEK is a target gene of lysine specific demethylase, LSD1 and repressed upon LSD1 knock-down (Scoumanne *et al*., 2007). Different roles of DEK in various signal transduction pathways have been implicated recently including the modulation of apoptosis through HAT inhibitory activity and DNA damage repair through ADP-ribosylation (Kappes *et al*., 2008; Lee *et al*., 2008).

In this study, we have identified and characterized the differentially expressed genes (DEGs) whose expressions were altered by proto-oncogene DEK overexpression using a differential display technique which is based on annealing control primers (ACPs) (Hwang *et al*., 2003). We have demonstrated that DEK is able to regulate certain number of genes that play major roles in apoptosis pathway.

## MATERIALS AND METHODS

### Construction of plasmids

The coding sequence of DEK was PCR-amplified with ligation of either a 5' BamHI site-introduced primer (5'-CGCGGATCCGCGATGTCCGCCTCGGCCCCTGCT-GCG-3') and a 3' EcoRI site-introduced primer (5'-CGC-GAATTCGCGTCAAGAAATTAGCTCTTTTAC-3'). For the eukaryotic expression construct of DEK, PCR product was cloned into the cloning site of pCMX PL1 vector, respectively. Sequences of construct surrounding the

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cloning sites were verified by automated sequencing.

### Cell culture and transient transfection

HeLa cells were grown in Dulbecco's modified Eagle's medium (Gibco) containing 10% heat-inactivated fetal bovine serum (Gibco) and 0.05% penicillin–streptomycin (Gibco) at 37°C with 5% $CO_2$ in humidified air. HeLa cells were seeded at $2\times10^5$ cells/well in 6-well dish and transient transfection to HeLa cells were performed using Lipofectamine 2000 (Invitrogen) with vector control pCMX PL1 (1 μg) and pCMX PL1-DEK (1 μg) for identification of differentially expressed genes and screening of apoptosis related genes.

### RNA isolation

Total RNA was isolated from untreated, control vector, and DEK transfected HeLa cells for ACP-based PCR analysis and multiplex PCR analysis. Total RNA extraction was carried out using TRIZOL reagent (Invitrogen), according to the manufacturer's instructions.

### Annealing control primer (ACP)-based GeneFishing™ PCR

For identification of differentially expressed genes (DEGs) from DEK overexpressed cells, which were screened by the ACP-based PCR method (Kim *et al*., 2004) using the GeneFishing™ DEG service (Seegene, Korea). Briefly, total RNA was treated with DNase I at room temperature for 15 min. For the synthesis of first-strand cDNAs, DNase I-treated total RNAs were reverse-transcribed by M-MLV reverse transcriptase. Reverse transcription was performed for 1.5 hr at 42°C in a reaction mixture containing 3 μg of the total RNA, 4 μl of 5× buffer, 5 μl dNTPs (each 2.5 mM), 2 μl of dT-ACP1 (10 μM) primer (Table I). Second-strand cDNA synthsis was performed using 1 μl of dT-ACP2 (1 μM), 1 μl of 20 arbitrary ACPs (Table I), and 2× Master Mix (Seegene, Korea). After second-strand cDNA synthesis was completed, this was

**Table I.** Primer sequences used in cDNA synthsis and ACP-based PCR

| Primer purpose | Primer name | Sequence (5'-3') |
|---|---|---|
| Reverse Transcription | dT-ACP1 | CTGTGAATGCTGCGACTACGAT I I I I I$(T)_{18}$ |
| GeneFishing PCR (reverse primer) | dT-ACP2 | CTGTGAATGCTGCGACTACGAT I I I I I$(T)_{15}$ |
| GeneFishing PCR (forward primer) | ACP 1 | GTCTACCAGGCATTCGCTTCAT I I I I IGCCATCGACC |
| | ACP 2 | GTCTACCAGGCATTCGCTTCAT I I I I IAGGCGATGCC |
| | ACP 3 | GTCTACCAGGCATTCGCTTCAT I I I I ICCGGAGGATG |
| | ACP 4 | GTCTACCAGGCATTCGCTTCAT I I I I IGCTGCTCGCG |
| | ACP 5 | GTCTACCAGGCATTCGCTTCAT I I I I IAGTGCGCTCG |
| | ACP 6 | GTCTACCAGGCATTCGCTTCAT I I I I IGGCCACATCG |
| | ACP 7 | GTCTACCAGGCATTCGCTTCAT I I I I ICTGCGGATCG |
| | ACP 8 | GTCTACCAGGCATTCGCTTCAT I I I I IGGTCACGGAG |
| | ACP 9 | GTCTACCAGGCATTCGCTTCAT I I I I IGATGCCGCTG |
| | ACP 10 | GTCTACCAGGCATTCGCTTCAT I I I I ITGGTCGTGCC |
| | ACP 11 | GTCTACCAGGCATTCGCTTCAT I I I I ICTGCAGGACC |
| | ACP 12 | GTCTACCAGGCATTCGCTTCAT I I I I IACCGTGGACG |
| | ACP 13 | GTCTACCAGGCATTCGCTTCAT I I I I IGCTTCACCGC |
| | ACP 14 | GTCTACCAGGCATTCGCTTCAT I I I I IGCAAGTCGGC |
| | ACP 15 | GTCTACCAGGCATTCGCTTCAT I I I I ICCACCGTGTG |
| | ACP 16 | GTCTACCAGGCATTCGCTTCAT I I I I IGTCGACGGTG |
| | ACP 17 | GTCTACCAGGCATTCGCTTCAT I I I I ICAAGCCCACG |
| | ACP 18 | GTCTACCAGGCATTCGCTTCAT I I I I ICGGAGCATCC |
| | ACP 19 | GTCTACCAGGCATTCGCTTCAT I I I I ICTCTGCGAGC |
| | ACP 20 | GTCTACCAGGCATTCGCTTCAT I I I I IGACGTTGGCG |

*poly(I), The polydeoxyinosine Linkers and representation of deoxyinosine.

subjected to second-stage PCR amplification. The amplified PCR products were separated in 2% agarose gel stained with ethidium bromide.

### Cloning and sequencing

Differentially expressed bands were purified from gels using the GENECLEAN® II Kit (Q-BIO gene) and were cloned into TOPO TA cloning vector (Invitrogen) according to the manufacturer's instructions. Sequencing of these clones was performed using ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). The DNA sequence of each gene was analyzed by searching for similarities using the BLAST at the National Center for Biotechnology Information GenBank.

### Apoptosis-related gene muliplex PCR

Multiplex PCR was performed using the Human apoptosis gene group (Cat. No. MH1900) of GeneXP™ service (Seegene, Korea). Briefly, 0.5 μg of total RNA were reverse-transcribed for the first-strand cDNA synthesis with 1 μl oligo-dT-ACP (Seegene, Korea) and M-MLV reverse transcriptase. The reaction mixture were containing 1 μl of RNase inhibitor, 4 μl of 5× RT buffer, 2 μl of dNTP (each 2.5 mM), 2 μl of 1 M DTT. The synthesized cDNAs were used as templates of muliplex PCR using the following condition: one cycle at 94°C for 15 min, 40 cycles of (94°C for 30 sec, 63°C for 1 min 30 sec, and 72°C for 1 min 30 sec), and then on final cycle at 72°C for 10 min with 5× Human APOP-X primer mix (Seegene, Korea). Housekeeping genes (ACTB, B2M, GAPD, SDHA, and RPL13A) were used as internal controls.

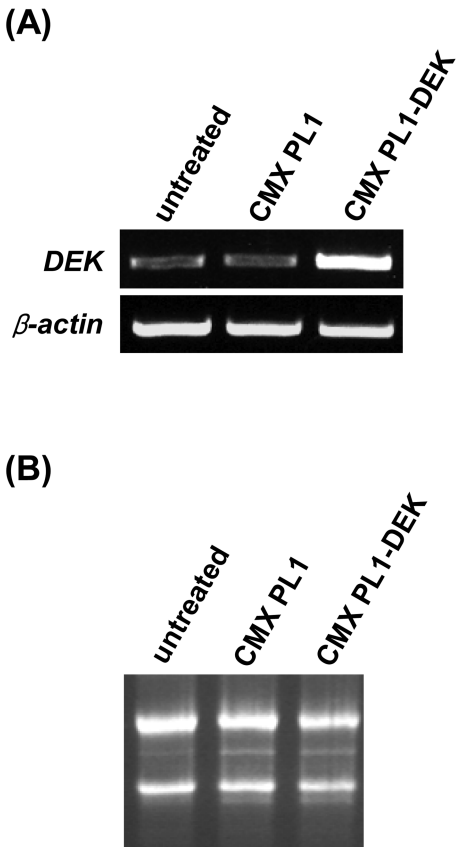


**Fig. 1. DEK overexpression in the transfected cells and isolation of RNA** (A) Overexpression of DEK in the HeLa cells was confirmed by RT-PCR using DEK specific primers. The β-actin was used as a control for equal RNA amounts. (B) Electrophoresis of total RNA from each sample (untreated, CMX PL1, and CMX PL1-DEK transfected cells).

## RESULTS AND DISCUSSTION

### Differentially expressed genes by DEK overexpression

To identify genes that are differentially expressed in the presence of DEK overexpression, we compared the mRNA expression profiles of untreated, CMX PL1 control, and CMX PL1-DEK transfected HeLa cells. First, overexpression of DEK in HeLa cell compared to the untreated and CMX PL1 empty vector transfected was confirmed by RT-PCR (Fig. 1A). Total RNA from each cells for ACP based PCR were compared (Fig. 1B). ACP technology based on the unique tripartite structure of a specific oligonucleotide primer, which has 3'- and 5'- distinct portions separated by a regulator, specifically targets sequence hybridization to the template via a polydeoxyinosine linker.

Using a combination of 20 arbitrary primers of two anchored oligo (dT) primers of ACP-based GeneFishing PCR kit, we isolated 11 DEGs in DEK overexpressed HeLa cell (Fig. 2). Among the 11 DEGs analyzed, 4 DEGs (DEG2, 4, 7, and 11) which showed significant change in their expression were cloned and sequenced for identification. BLAST search revealed that some of the cloned genes or ESTs had significant sequence similarities with known genes in the GenBank (Table II).

One of the differentially expressed genes in DEK overexpressed cell (DEG 4, Fig. 2 and Table II), clusterin, is ubiquitously expressed in various cells and tissues (de Silva *et al*., 1990) and it is overexpressed in several human cancers such as prostate (Kadomatsu *et al*., 1993), breast (Redondo *et al*., 2000), and ovarian cancer (Hough *et al*., 2001). Clusterin has been reported to be involved in various biological processes such as carcinogenesis, tumor growth, and apoptotic cell death (Shannan *et al*., 2006). Recent report shows that knock down

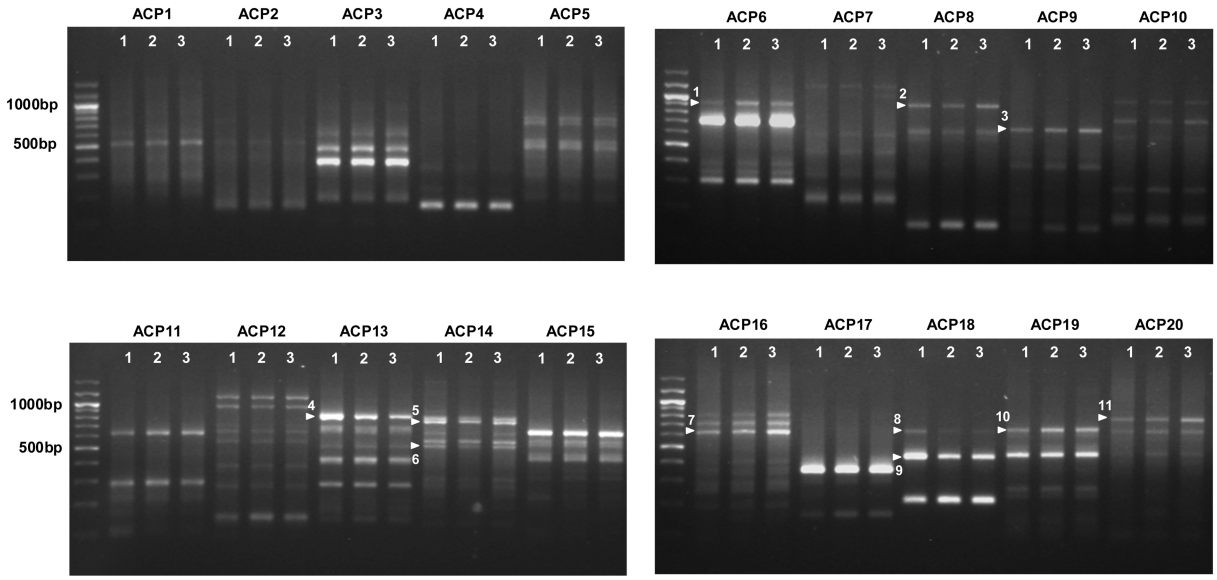


**Fig. 2. Annealing control primer (ACP)-based PCR for the identification of differentially expressed genes (DEGs) from DEK overexpressed HeLa cells.** Using 20 ACPs (as a arbitrary forward primer) and a dT-ACP2 (as a reverse primer), 11 differentially expressed genes (white arrowheads) were identified in DEK overexpressed cells. Four of them were cloned and sequenced for identification. (1, untreated; 2, CMX PL1; 3, CMX PL1-DEK).

**Table II.** Identification of differentially expressed genes (DEGs) in DEK transfected HeLa cells

| DEG No. | Accession No. | Gene | Identities |
|---|---|---|---|
| DEG 2 | BC019260.1 | Homo sapiens fibrillarin, mRNA (cDNA clone MGC:2347 IMAGE:3504198), complete cds Length=1163 | 806/808 (99%) |
| DEG 4 | NM_001831.2 | Homo sapiens clusterin (Complement lysis inhibitor, SP-40,40, sulfated glycoprotein 2, testosterone-repressed prostate message 2, apolipoprotein J) (CLU), transcript variant 1, mRNA Length=2859 | 743/743 (100%) |
| DEG 7 | BC094774.1 | Homo sapiens, polypeptide 4, 90kDa, mRNA (cDNA clone MGC:104675 IMAGE:4830453), complete cds Length=3082 | 508/510 (99%) |
| DEG 11 | NM_001026.3 | Homo sapiens ribosomal protein S24 (RPS24), transcript variant 2, mRNA Length=593 | 468/470 (99%) |

of clusterin transcripts via siRNA results in growth retardation and increase of apoptosis, and induces cell sensitization to genotoxic and oxidative stress (Trougakos *et al*., 2004). On the contrary, it has been suggested that clusterin plays an important role in inhibiting apoptosis in human cancer cells by interfering with pro-apoptotic protein Bax activation, thereby inhibiting cytochrome C release (Zhang *et al*., 2005). Clusterin was down-regulated in DEK overexpressed cells compared with untreated and CMX PL1 transfected cells. These results suggest that HAT inhibitory activity of proto-oncogene protein DEK negatively effects on promoter and/or transcriptionally regulated region of clusterin as a transcriptional regulator. Our previous report has shown that DEK overexpression in *Drosophila in vivo* system and HeLa cells induce the apoptotic cell death (Lee *et al*., 2008). Therefore, down-regulation of clusterin in DEK overexpressed cells may promote the apoptotic cell death through HAT inhibitory activity in their promoter.

The ribosomal protein S24 (RPS24), was up-regulated in DEK overexpressed cells (DEG 11, Fig. 2 and Table II). Ribosomal proteins are a major component of ribosomes, therefore play critical roles in protein biosynthesis. Also, it was involved in various cellular processes such as replication, transcription, RNA processing, and DNA repair. Recently it has been shown that ribosomal proteins were significantly increased in normal mucosa, whereas markedly decreased in colorectal cancer (Kasai *et al*., 2003). We found that RPS24 expression was significantly increased by DEK overexpression, suggesting

that it may be involved in DEK-mediated apoptosis through involvement of cellular processes via their extraribosomal functions.

Fibrillarin is a component of the nucleolar small nuclear ribonucleoprotein, which has an important role in pre-rRNA processing and ribosome assembly during ribosomal biogenesis (Lafontaine *et al*., 2000). Recent report observed that fibrillarin would play a critical role in the maintenance of nuclear shape and cellular growth in human cells and mouse (Newton *et al*., 2003; Amin *et al*., 2007). In our study, fibrillarin expression was slightly increased when DEK was overexpresed (DEG 2, Fig. 2 and Table II). This increase may be due to the DEK-mediated apoptosis for cell protection. General transcription factor IIIC (TFIIIC) (DEG 7, Fig. 2 and Table II) expression was highly increased, which has HAT activity with specificity towards histone H3 (Hsieh *et al*., 1999). Elevation of TFIIIC expression level in DEK transfected cell suggests that the effect might be a response against elevated histone deacetylation level by HAT inhibitory activity of DEK and towards the maintenance of histone acetylation level.

**Screening of apoptosis related genes using multiplex PCR**

We have previously reported the role of DEK in caspase-mediated apoptosis in *Drosophila* and human carcinoma cell using genetic and biochemical analyses (Lee *et al*., 2008). DEK overexpression induces caspase-dependent apoptotic cell death in *Drosophila* as well as HeLa cell through capase-9 and -3 activation. To further elucidate the role of DEK in apoptosis pathway, we performed screening of expression patterns of apoptosis related genes using GeneXP analysis (Seegene, Korea) (Fig. 3). DEK overexpressed cells showed substantial increase of certain caspase series such as caspase-3, -7, and -10. In addition, pro-apoptotic protein bid was enhanced its expression level in DEK overexpressed cells, which triggers apoptosis by cytochrome C release and mitochondria damage such as loss of the membrane potential. Taken together, elevation of caspase-3, -7, and -10 and bid expression reinforce our previous report that DEK induces apoptotic cell death through caspase-dependent pathway.

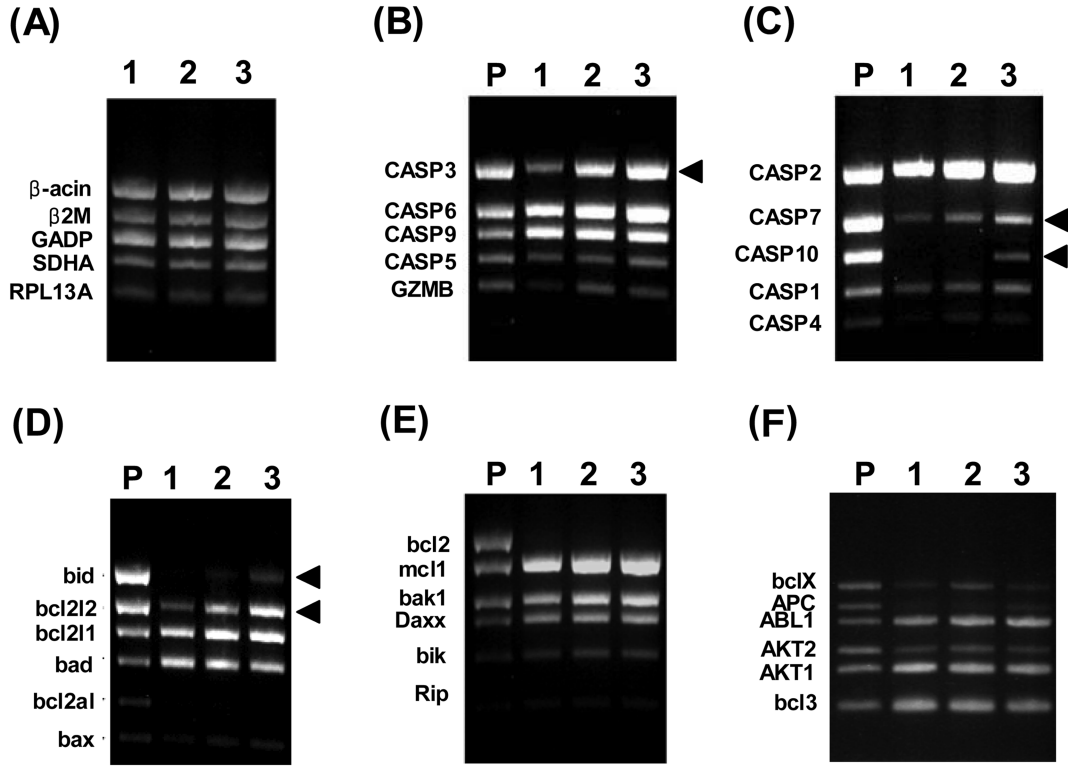


**Fig. 3. Screening of apoptosis related genes using multiplex PCR.** (A) House keeping genes (B2M, β-2-microglobulin; GAPD, glyceraldehydes-3-phosphate dehydrogenase; SDHA, succinate dehydrogenase complex, subunit A; RPL13A, ribosomal protein L13a) were used as controls for equal RNA amounts in these analysis. Arrowheads indicate the bands showing significant changes in their expression. (P, positive control; 1, untreated; 2, CMX PL1; 3, CMX PL1-DEK).

## ACKNOWLEDGEMENTS

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