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Master's thesis

AP endonuclease regulates DSC2 expression in colon and cervix cancer cell lines

Graduate school of Chosun University

Department of Bio-Material Engineering

NGUYEN THUAN LOI

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AP endonuclease는 대장암과 자궁경부암에서 DSC2발현을 조절한다

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NGUYEN THUAN LOI

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Advisor: Professor Ho Jin You

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Department of Bio-Material Engineering

NGUYEN THUAN LOI

NGUYEN THUAN LOI 의 석사학위 논문을 인준함

Chusun University: Professor In Youb Chang

Chusun University: Professor Jea Yeoul Jun

Chusun University: Professor Ho Jin You

June 2008

Graduate school of Chosun University

NGUYEN THUAN LOI

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Nguyen Thuan Loi

Advisor: Prof. Ho Jin You, Ph.D., M.D.

Department of Bio material Engineering

Graduate School of Chosun University

ABSTRACT

The major role of human APE in the DNA base excision repair pathway is responsible for repair of apurinic/apyrimidinic site in DNA. APE also has been reported to be involved in the process in cancer and apoptosis.

I have found APE regulates the expression of RB1, DSC2 and LOX in GM00637 and GM00637/APE cell lines through DNA microarray. RB1, LOX and DSC2 were strongly expressed in DLD1, DU145 and Ca Ski cancer cell lines, but they did not expressed in DM533 and PC-3 cancer cell lines in which APE was weakly expressed. Moreover, DSC2 has been highly expressed in DLD1 and Ca Ski cancer cell lines. Blocking APE by APE siRNA dramatically decreased the expression of DSC2 which

has been involved in apoptosis and cell adhesion in colon cancer.

Take together, the correlation with APE and DSC2 suggests that they may be an important role of apoptosis and process in cancer biology.

I. INTRODUCTION

The human DNA repair enzyme apurinic/apyrimidinic endonuclease (APE, also known as Hap1, Apex and Ref-1) is a multifunctional protein in the DNA base excision repair (BER) pathway that is responsible for repair of apurinic/apyrimidinic (AP) sites in DNA.

The process of programmed cell death or apoptosis involves activation of a specific cellular pathway that results in death of the cell both as a response to cell injury and as part of normal development. Details of the factors involved in apoptosis and their regulation have been the focus of intense interest in recent years (Hockenbery., 1995; Vang, E a. K Molecularthanatopsis., 1996.). Both DNA repair and apoptosis function to protect the organism from the sequelae of serious cellular damage. DNA repair systems serve to prevent deleterious genetic damage from being passed on to the next cellular division, and apoptosis eliminates more extensively damaged cells that are beyond the normal repair mechanisms (Kent A. Robertson et al., 1997).

The human APE has been associated with other functions, it may be an anti-apoptotic protein associated with cell differentiation (Luciana B. Chiarini et al., 2000) or stopped cell proliferation and activated apoptosis (Fung,H. et al., 2005).

In addition, APE regulatory function of APE1 with many gene expressions has been demonstrated. Here, we also investigate genes regulate APE by DNA microarray.

The DNA microarray is a high-throughput technology used in molecular biology and in medicine. It consists of an arrayed series of thousands of microscopic spots of DNA oligonucleotides, called features that each containing picromoles of a specific DNA sequence. These can be a short section of a gene or other DNA element that are used as probes to hybridize a cDNA or cRNA sample (called target) under high-stringency conditions. Probe-target hybridization is usually detected and quantified by fluorescence-based detection of fluorophore-labelled target to determine relative abundance of nucleic acid sequences in the target.

In standard microarrays the probes are bound to a solid surface by covalent attachment to a chemical matrix (via epoxy-silane, amino-silane, lysine, polyacrylamide or others). The solid surface can either be glass or a silicon chip, in which case they are commonly known as gene chip or colloquially Affy Chip when an Affymetrix chip is used. Some microarray platforms, such as Illumina, use microscopic beads, instead of the large solid support (glass or treated silicon) used in traditional microarrays. DNA arrays are different from other types of microarray only in that they either measure DNA or use DNA as part of its detection system.

DNA microarrays can be used to measure changes in expression levels or to detect of SNPs (see Types of arrays section). There are not only different microarray applications, but also differences in the fabrication and workings of the microarrays, which as a result differ in accuracy, efficiency, and cost (see fabrication section). Furthermore, additional factors important to microarray experiments are the experimental design and analysis methods of the data.

Desmocollin 2 is one protein encoded by this gene is a calcium-dependent glycoprotein that is a member of the desmocollin subfamily, desmosome type of cell-cell junctions are members of the cadherin family of cell adhesion molecules (Goodwin et al., 1990; Holton et al., 1990; Koch et al., 1990, 1991b; Collins et al., 1991; Mechanic et al., 1991; Nilles et al., 1991; Parker et al., 1991; Wheeler et al., 1991). These desmosomal family members, along with the desmogleins, are found primarily in epithelial cells where they constitute the adhesive proteins of the desmosome cell-cell junction and are required for cell adhesion and desmosome formation. The desmosomal family members are arranged in two clusters on chromosome 18, occupying less than 650 kb combined.

Desmoglein 2 is a member of desmosome family is regulator of apoptosis in the intestinal epithelium (Porfirio Nava et al., 2007). Desmocollin also play important role in colorectal cancer (Khan K et al., 2006). In addition, DSC2 is ubiquitously expressed in epithelial tissues and the heart of adult mice and from the blastocyst stage of

development (Lorimer JE et al., 1994).

In this study, by DNA microarray I have found APE regulates some genes that are expressed in cancer cell lines. One of them is DSC2. It belongs to desmosome family has required for cell adhesion and may be regulator of apoptosis.

II. MATERIALS AND METHODS

1. DNA microarray.

1.1 RNA preparation

Total RNA was extracted using Trizol (Invitrogen Life Technologies, Carlsbad, USA), purified using RNeasy columns (Qiagen, Valencia, USA) according to the manufacturers' protocol. After processing with DNase digestion, clean-up procedures, RNA samples were quantified, aliquot and stored at -80°C until use. For quality control, RNA purity and integrity were evaluated by denaturing gel electrophoresis, OD 260/280 ratio, and analyzed on Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, USA).

1.2. Labeling and purification

Total RNA was amplified and purified using the Ambion Illumina RNA amplification kit (Ambion, Austin, USA) to yield biotinylated cRNA according to the manufacturer's instructions. Briefly, 550 ng of total RNA was reverse-transcribed to cDNA using a T7 oligo (dT) primer. Second-strand cDNA was synthesized, in vitro transcribed, and labeled with biotin-NTP. After purification, the cRNA was quantified using the ND-1000 Spectrophotometer (NanoDrop, Wilmington, USA).

*1.*3. *Hybridization and data export*

750 ng of labeled cRNA samples were hybridized to each mouse-6 expression bead array for 16-18 h at 58°C, according to the manufacturer's instructions (Illumina, Inc., San Diego, USA). Detection of array signal was carried out using Amersham fluorolink streptavidin-Cy3 (GE Healthcare Bio-Sciences, Little Chalfont, UK) following the bead array manual. Arrays were scanned with an Illumina bead array Reader confocal scanner according to the manufacturer's instructions. Array data export processing and analysis was performed using Illumina BeadStudio.

1.4. Raw data preparation and Statistic analysis

The quality of hybridization and overall chip performance were monitored by visual inspection of both internal quality control checks and the raw scanned data. Raw data were extracted using the software provided by the manufacturer (BeadStudio v. 2.1.12). Array data were filtered by detection p-value < 0.05 (similar to signal to noise) in at least 50% samples (we applied a filtering criterion for data analysis; higher signal value was required to obtain a detection p-value < 0.05). Selected gene signal value was transformed by logarithm and normalized by quantitative method. Significantly expressed genes were selected by fold-change (greater or equal to 2), was visualized by hierarchical cluster analysis. Hierarchical clustering with complete linkage and Euclidean distance were visualized by ArrayAssist® (Stratagene, La Jolla,

USA). All data analysis and visualization of differentially expressed genes was conducted using ArrayAssist® (Stratagene, La Jolla, USA). Biological pathway and ontology-based analysis were performed by using Panther database (http://www.pantherdb.org).

2. Cell culture

GM00637 (human fibroblast cell) from Cornell Institute for Medical Research and DU145 (human prostate cancer) were cultured in EMEM (Gibco, Gland Island, NY, USA) containing 10%FBS, 100U/ml penicillin, and 100μg/ml streptomycin at 37℃ in a humidified atmosphere of 5% CO_2. DLD1 (human colon cancer cell), DMS53 (human lung small cell carcinoma), PC-3 (human prostate carcinoma) and Ca Ski (human Cervix cancer) cell lines were cultured in RPMI 1640 (Gibco, Gland Island, NY, USA) containing 10%FBS, 100U/ml penicillin, and 100μg/ml streptomycin at 37℃ in a humidified atmosphere of 5% CO_2.

3. Plasmid constructs and Transfection

The APE were amplified by RT-PCR using the APE oligo primer (forward primer; 5-ATG CCG AAG CGT GGG AAA AAG-3, reverse primer; 5- CA CAG TGC TAG GTA TAG GGT GA-3). After confirming the sequence, the APE cDNA was cloned into a pcDNA3.1/V5-His-Topo mammalian expression vector (Invitrogen, K4800-1,

Carlsbad, USA), which was driven by the CMV promoter (Invitrogen, Carlsbad, USA). After confirming the sequence, the APE cDNA was cloned into a pcDNA3.1/V5-His-Topo mammalian expression vector, which was driven by the CMV promoter (Invitrogen, Carlsbad, USA). The APE construct was transfected into cells using the Lipofectamine 2000 (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions. After transfection, cells were incubated with complete medium containing 400ug/ml G418 for 5 weeks. The cell clones resistant to G418 were isolated and analyzed.

4. Reverse transcriptase–polymerase chain reaction

RNA extraction was conducted using the RNA-STAT-60 according to the manufacturer's instructions (TEL-TEST, Inc., Friendswood, TX). Briefly after homogenizing cells in the RNA-STAT-60, the homogenate was mixed with chloroform (5:1v/v) vortex vigorously for 15s, and then centrifuged at 13,000 rpm for 15min at 4°C. The RNA present in the upper colorless aqueous phase was precipitated by adding isopropylalcohol, which was washed twice with 70% ethanol and then air-dried for 10 min. The RNA was then resuspended in DEPC. RNA were prepared and stored at -70°C until needed. 3μg of the total RNA was reverse-transcribed using an M-MLV cDNA synthesis system (Invitrogen, Carlsbad, USA), and the reverse-transcribed DNA was subjected to PCR. 50ng of cDNA was subjected to a polymerase chain

reaction (PCR). The profile of replication cycles was denaturation at 94°C for 30 seconds, annealed at 58°C for 30 seconds, and polymerized at 72°C for 1 min. In each reaction, the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as the internal control. The PCR products were resolved on 1.3 % agarose gels, stained with ethidium bromide, and then photographed.

5. Western blotting

The cell were washed with phosphate-buffered saline (PBS) and lysed on ice for 10min in the M-PER mammalian protein Extraction Reagent (PIERCE, USA) added protease Inhibitor Cocktail tablet (Roch). After incubation, extracts were vortexed for 5min and centrifuged at 13,000rpm for 15min. The supernatant was diluted with 5X SDS-sample buffer and boiled. After cellular protein concentrations were determined using the dye-binding microassay (Bio-Rad, Hercules, CA), and 40ug of protein per lane were separated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). After SDS-PAGE, the proteins were transferred onto Hybon ECL membranes (Amersham Biosciences, Piscataway, NJ). After electroblotting, the membranes were blocked by 5% skim-milk in Tris buffer saline containing 0.05% Tween-20 (TBST, 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1 % Tween-20) at room temperature for 2 hours. The membranes were rinsed with TBS-T and then incubated with appropriate primary antibodies in TBS-T at 4℃ overnight. All antibodies used in this study are α-

tubulin, monoclonal antibody DSC-2 (Santa Cruz, USA). We followed manufacturer's protocol for dilution of all primary antibodies. The membranes were then washed, incubated with the biotinylated secondary antibodies (1:500) in a blocking buffer for 2 hours at room temperature, and washed again. The blotted proteins were developed using an enhanced chemiluminescence detection system (iNtRON, Biotech, Seoul, Korea).

6. Statistical Analysis

All experiments were repeated at least three times to ensure reproducibility. Data in all experiments are represented as mean ± S.E. Statistical comparisons were carried out using two-tailed Student's *t* test. *p* values <0.05 were considered to be statistically significant.

III. RESULTS

1. Genes induced with APE by DNA microarray screening.

To identify the genes regulate with AP endonuclease, I used DNA microarray (Figure.1). Significantly expressed genes were selected by fold-change was visualized by hierarchical cluster analysis. I have found that in APE-induced cell RB1and DSC2 were highly up-regulated, but LOX was down-regulated.

2. APE regulates gene expression.

To confirm the genes screening by DNA microarray regulated by APE, I have designed primers and performed PCR in GM00637(wild-type) and GM00637/APE overexpression. Analysis RT-PCR result using these specific primers showed that the expression level of these genes was induced dramatically in APE-overexpressed cell line. RB1 and DSC2 were highly expressed in GM00637/APE overexpressed cell lines, but LOX is down-regulated (Figure.2).

3. APE regulated genes expression in cancer cell lines

To study APE-induced genes in cancer cell lines, I checked the gene expression including APE, RB1, LOX and DSC2 in DLD1, DU145, Ca Ski, DMS53 and PC-3 cancer cell lines. APE, DSC2, RB1 and LOX are strong expression in DLD1, DU145, Ca Ski cell, but their expressions are weak in DMS53 and PC-3.

4. APE regulates DSC2 expression in cancer cell lines

As it was shown in Fig.2 and Fig.3, DSC2 was dramatically up-regulated by APE in GM00637/APE, DLD1 and Ca Ski cancer cell lines. To confirm whether APE is indeed involved in the DSC2 expression in DLD1 and Ca Ski cancer cell lines, I performed PCR with APE siRNA-treated and negative siRNA-treated cell lines. I found DSC2 was strongly regulated by APE (Figure.4A).

Expression of the DSC2 was verified by western blot analysis of protein lysates prepared from transfected cells. The protein expressed by DSC2 significantly higher levels in negative siRNA-treated than in APE siRNA-treated cell line. I found that down-regulated APE decreased DSC2 expression in DLD1 and Ca Ski cancer cell line (Figure.4B).

Table1. The primers for PCR

Primer	Sequence	Tm (℃)
APE sence APE antisence	5'-ATG CCG AAG CGT GGG AAA AAG-3' 5'-CAC AGT GCT AGG TAT AGG GTG A- 3'	58
DSC2 sence DSC2 antisence	5'-TCC ATT TTC CAG AGA GGC-3' 5'-AGG ATC AAC CGC AAC AAT-3'	58
RB1 sence RB1 antisence	5’-TGT CTT TAT TGG CGT GCG CT-3’ 5’-TGG GTC TGG AAG GCT GAG GT-3’	58
LOX sence LOX antisence	5’-ACA TCT AGA GCC CGC GAA GC-3’ 5’-GGG AAA TCT GAG CAG CAC CC-3’	58
GAPDH sence GAPDH antisence	5'-TGA CCA CAG TCC ATG CCA TC-3' 5'-TTA CTC CTT GGA GGA GGC CAT GT-3'	58

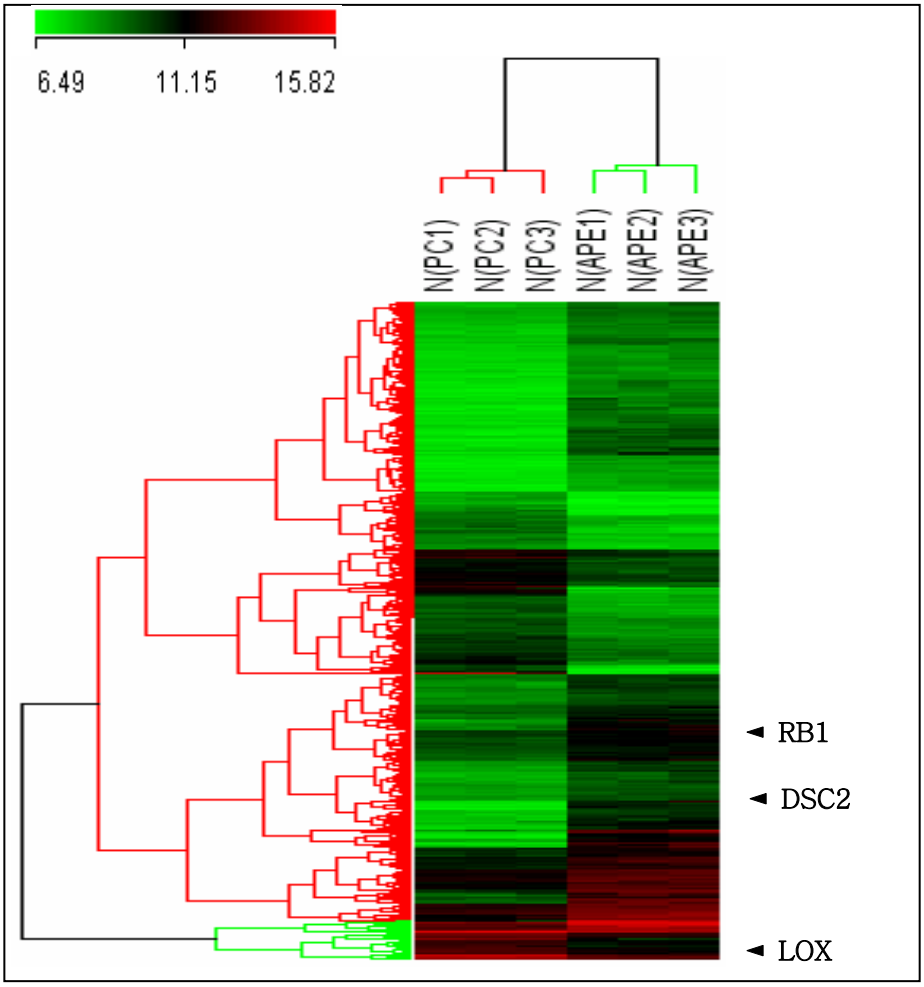

Fig.1. Gene Expression Analysis of APE-expressed cell line

Basal gene expression differences between GM00637/pcDNA3 and APE-expressed GM00637. Data presented in the 728 rows correspond to 178 unique genes most differentially expressed between the two lines that were selected (p-value<0.05). Elements were further filtered for being at least 2-fold different between the lines and analyzed.

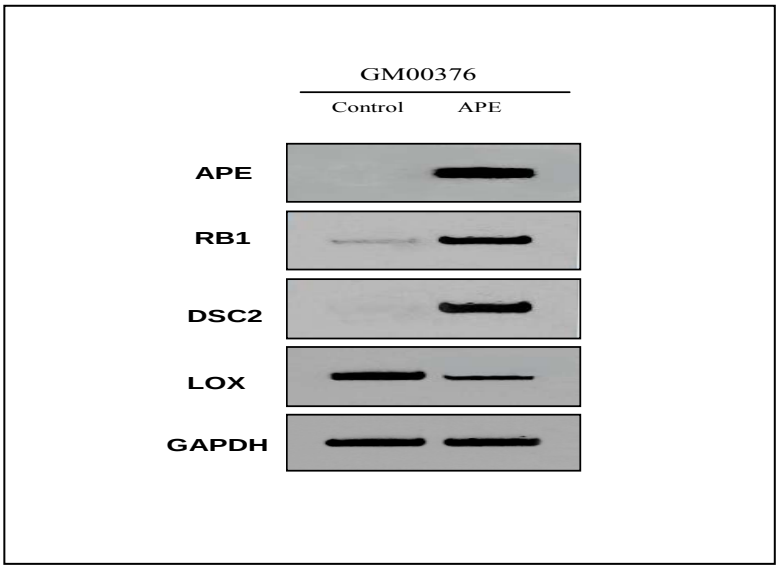

Fig.2. APE regulated genes expression in GM00637 and GM00637/APE cells

Expression of genes by PCR was compared between GM00637 and GM00637/APE cells. Amplified DNA products were separated on a 1.3% standard agarose gel and stained with ethidiumbromide.

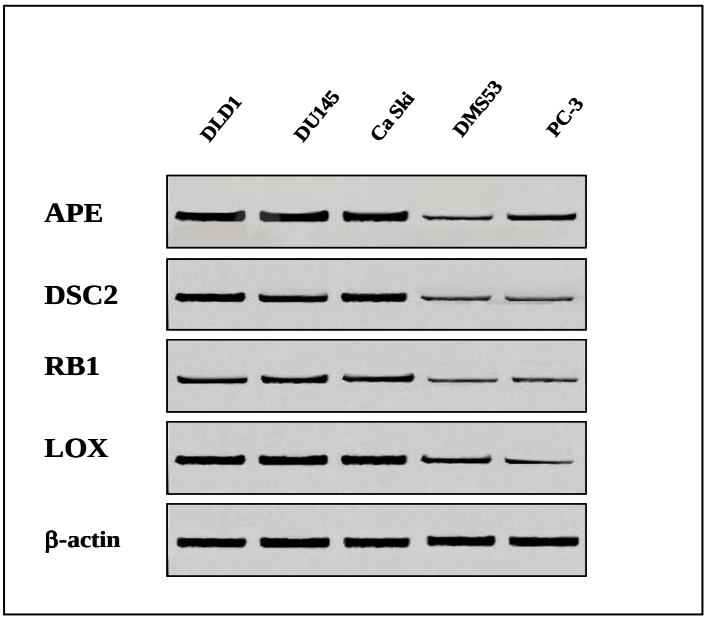

Fig.3. APE regulated genes expression in cancer cell lines

Expression of genes by PCR was compared in DLD1, DU145, Ca Ski, DMS53, PC-3 cancer cell lines. Amplified DNA products were separated on a 1.3% standard agarose gel and stained with ethidium bromide.

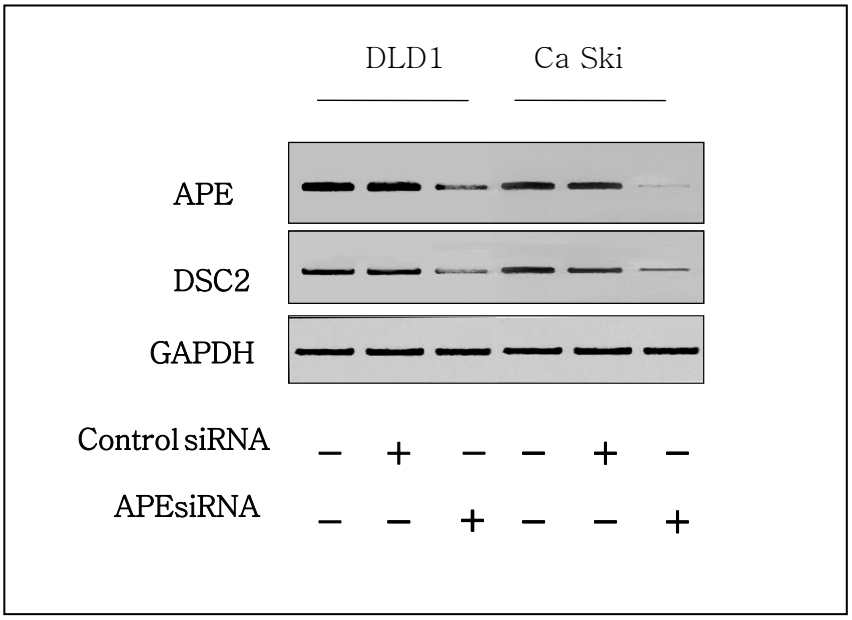

Fig.4A. APE regulates DSC2 expression in DLD1 and CaSki cancer cell lines

Expression of genes by PCR was compared in DLD1 and Ca Ski cancer cell lines. Amplified DNA products were separated on a 1.3% standard agarose gel and stained with ethidium bromide.

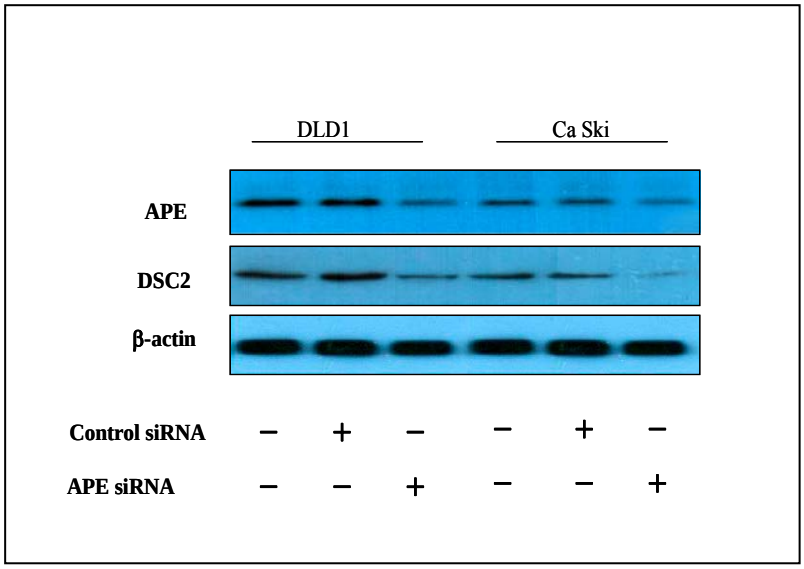

Fig.4B. APE regulates DSC2 protein expression in DLD1 and CaSki cancer cell lines

Protein levels of APE and DSC2 were confirmed in APE siRNA-transfected DLD1 and Ca Ski cell lines on immunoblot. β-actin was used as the loading control.

IV. DISCUSSION

Here, I have screened APE regulated RB1, DSC2 and LOX on microarray. Through PCR technique, I also confirm them in GM00637 and GM00637/APE overexpression cell line. APE up-regulates RB1 and DSC2 but down-regulates LOX (Fig.2). Their expressions have been checked in cancer cell lines.

I compared DSC2 expression in negative and APE Si-RNA-transfected DLD1 and Ca Ski cell lines. DSC2 gene and protein expression were inhibited by APE Si-RNA in both DLD1 and CaSki cancer cell lines.

According to the latest reports, APE has been involved in apoptosis. It may through DNA fragmentation during apoptosis (Akira Yoshida et al., 2003). The silencing of APE expression decreases apoptotic DNA fragmentation in DFF40/CAD-deficient cells (Yoshida.A et al., 2003). It may be a potent activator of p53 DNA binding in vitro (Gaiddon C et al., 1999).

DSC2 has known as one of the cadherin superfamily. It functions cell- cell junctions. DSC2 is a member of the desmocollin subfamily and it may play important role in apoptosis (Porfirio Nava et al., 2007).

In the present study, APE tightly regulates DSC2. DSC2 expression was inhibited in APE siRNA –treated DLD1 and CaSki transfected cell lines and highly expressed in control and negative cell lines. APE has been reported as an apoptosis inducer (Hall JL

et al., 2001; Wang N et al., 2001) and the silencing of APE expression decreases apoptotic process (Yoshida.A et al., 2003). Also, it has been reported that DSC2 is involved in apoptosis in colon cancer (Porfirio Nava et al., 2007).

Take together, I suggest that apoptosis signaling of APE may be involved in DSC2.

For the further study, I will check the apoptosis signaling with APE siRNA-treated and DSC2 overexpression in APE siRNA –treated cell lines.

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<국문초록>

AP endonuclease는 대장암과 자궁암 세포주에서 DSC2의 발현을 조절한다.

Nguyen Thuan Loi

(지도교수: 유호진)

조선대학교 일반대학원

생물신소재학과

DNA 염기제거수복과정에서 APE는 DNA에서 퓨린/피리미딘이 없어진 부위에 관여한다. 또한, APE는 암 생성 과정과 세포 자멸사에도 관여하는 것으로 보고 되어있다.

DNA microarray라는 기술로 APE를 발현시키는 GM00637세포의 DNA와 대조군 DNA를 분석한 결과, RB1, DSC2 그리고 LOX의 발현이 증가함을 찾아내었다. 이들 유전자들은 APE가 많이 발현되어있는 DLD1, DU145 와 Ca Ski 암 세포 주에서 발현이 현격히 증가되어있음을 확인할 수 있었고, 반면에 DMS53 와 PC-3 암 세포 주처럼 APE가 거의 발현되지 않는 세포 주들에서는 매우 약하게 발현되고 있음을 관찰하였다. 더욱이, APE siRNA를 사

용하여 DLD1 과 Ca Ski 암 세포 주에서 APE발현을 억제하였을 때 DSC2발현이 억제됨을 확인하였다.

따라서, APE는 대장암세포 자멸사와 세포부착에 관여하는것으로 알려진 DSC2의 발현을 조절하며, APE와 DSC2의 상호작용은 대장암과 자궁경부암과 밀접한 관련성이 있을 것이라 사료된다.

저작물 이용 허락서

학 과	생물신소재학과	학 번	20067745	과 정	석사
성 명	한글 :로이 응구엔 뚜안 영문 : NGUYEN THUAN LOI				
주 소	광주광역시 서석동 조선대학교 DNA 수복센터				
연락처	E-MAIL : anhloi123@yahoo.com				
논문제목	한글: AP endonuclease는 대장암과 자궁경부암에서 DSC2발현을 조절한다 영문: AP endonuclease regulates DSC2 expression in colon and cervix cancer cell lines				

본인이 저작한 위의 저작물에 대하여 다음과 같은 조건아래 조선대학교가 저작물을 이용할 수 있도록 허락하고 동의합니다.

- 다 음 -

1. 저작물의 DB구축 및 인터넷을 포함한 정보통신망에의 공개를 위한 저작물의 복제, 기억장치에의 저장, 전송 등을 허락함
2. 위의 목적을 위하여 필요한 범위 내에서의 편집·형식상의 변경을 허락함. 다만, 저작물의 내용변경은 금지함.
3. 배포·전송된 저작물의 영리적 목적을 위한 복제, 저장, 전송 등은 금지함.
4. 저작물에 대한 이용기간은 5년으로 하고, 기간종료 3개월 이내에 별도의 의사표시가 없을 경우에는 저작물의 이용기간을 계속 연장함.
5. 해당 저작물의 저작권을 타인에게 양도하거나 또는 출판을 허락을 하였을 경우에는 1개월 이내에 대학에 이를 통보함.
6. 조선대학교는 저작물의 이용허락 이후 해당 저작물로 인하여 발생하는 타인에 의한 권리 침해에 대하여 일체의 법적 책임을 지지 않음
7. 소속대학의 협정기관에 저작물의 제공 및 인터넷 등 정보통신망을 이용한 저작물의 전송·출력을 허락함.

동의 여부: 동의 (O) 반대 ( )

2008 년 8 월 일

저작자: Nguyen Thuan Loi (서명 또는 인)

조선대학교 총장 귀하