Identification of proteins and genes of transformed U87-MG cell related to radiosensitivty under hypoxia

Han Jo Kim

Department of Medical Science

The Graduate School, Yonsei University

Identification of proteins and genes of transformed U87-MG cell related to radiosensitivity under hypoxia

Directed by Professor Jong Eun Lee

The Master's Thesis
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This certifies that the Master's Thesis
of Han Jo Kim is approved.

Thesis Supervisor : Jong Eun Lee

[Kyung Ah Park: Thesis Committee Member#1)

[Jinsil Seong: Thesis Committee Member#2)

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Yonsei University

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This season of winter feels more likely cold and unusually longer than what I thought. I also felt that my living existence in graduate school, was longer and hard. However, I overcome with couple different peoples help and their lovely attentions. And all these precious experience will remain as memory of great opportunity. Although my mistakes and not enough stuffs, I truly thanks for professor Jong Eun Lee for guidance and inspiration. I wish to thank professor Kyung Ah Park and Won Taek Lee for the excellent proofreading and suggestions in my thesis. I would like to also thank professor Jin Sil Sung for great advice and suggestions in my thesis. And then I wish to acknowledge my lab colleagues, Jong Youl Kim , the one who has moral senses and righteousness. The man who has great honesty and strong leadership of Jae Hwan Kim, my belonging senior of Yoon Jeong Choi, my God mother of Su Kyung Ahn, always giving me helpful praise was Meizi Yang, Chin Hee Mun, Ji hee Kim, nice Sa Hyun Kim and hardworking Yong Woo Lee. I truly thanks for your convenience and when I go out to society of the world, then I will do my best and give effort for my best responsibility as much as all you guys expect me. The people who help me from America named Brother Tae Sik and Tae hee, and especially Hye Jin . My Mother, Father, Sister, Grandmother and Grandfather, once again I truly thank you and I truly appreciate for all the help that I received.

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ABSTRACT

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Malignant brain tumors, especially glioblastomas U87-MG cells expressing wild-type p53 are resistant to radiation therapy under hypoxic condition. Tumor-specific therapies that increased radiosensitivity under hypoxic cancer cells are clearly needed. Human papillloma virus-type16 E7, which inhibits pRb, can also abrogate growth arrest induced by DNA

damaging agent in cultured cells. HPV-16 E7 gene induced increased levels of p53 and p21 proteins after irradiation. The p53 gene is critical to monitoring DNA damage. Wild-type p53 induced a G1 arrest, DNA repair, and apoptosis after exposure to ionizing radiation and accumulated under hypoxic condition. We investigated that U87-MG cells expressing HPV-16 E7 undergo cell death more severely than U87-MG cells after irradiation and post-irradiation under hypoxic condition. We observed that significantly different proteins (113) using 2-D systems and identified several spots assuming related to cell death by maldi-analysis. and we identified three spots including TNF superfamily. Many of genes with increased expression in U87△E7 gene encode proteins that are involved in cell cycle or that have been reported to affect cell proliferation and the genes with decreased expression were stress response proteins. Especially the expression of heat shock protein 70 was decreased in U87△E7 cells after irradiation under hypoxia. Bax and p53 that have been reported as pro-apoptotic proteins were increased in U87△E7 cells after under hypoxia. In this study, we demonstrated that HPV-16 E7 gene transfection altered the experssion of several apoptosis-associated proteins after irradiation under hypoxia. These results might suggest that the expression of HPV-16 E7 oncogene can induce the vulnerability of U87-MG cells to radiotherapy.

Key words : E7 gene, U87-MG, 2-D, Microarray, hypoxia, irradiation.

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I. INTRODUCTION

Gliomas are the most common human brain tumors and are divided into four stages by WHO classification scheme. Less malignant gliomas are defined as grades I and II, whereas grade III and grade IV are malignant. Although both grades III and IV are malignant, the prognoses for these tumors are quite different.[1,2] Malignant brain tumors, especially glioblastomas, are notorious for their resistance to therapy. Apart from intrinsic properties of glioma cells involved in the resistance to radiotherapy and chemotherapy, the presence of hypoxia in these tumors may be an important factor in determining the resistance to radiation treatment.[4,5,6] The glioblastoma cell

lines, U87 MG expressing wild-type p53and U373 MG expressing mutant-type p53, have been investigated on the contribution of p53 to the radiationresponse.[6,7,8] Radiation-induced DNA damage and cytotoxicity are central issues for understanding tumorigenesis and for optimizing radiation therapy. The relationship between p53 gene expression and cellular radiosensitivity, however, is unclear.[9,10,11] Increased radiosensitivity, and no apparent difference in radiosensitivity have all been associated with p53 function in various studies. The human papillomaviruses (HPVs) are small DNA-containing viruses, which exhibit a specific tropism for epithelial cells, and infection with these HPV types confers a high-risk for the development of malignant disease. The most common of these high-risk HPV types, HPV-16 and HPV-18, have also been shown to encode transforming and immortalizing activities in cultured cells.[12,13,14] The HPV-16 E7 protein has been ascribed to its ability to bind to, and down regulate, the retinoblastoma (Rb) gene product and leads to stabilization of the p53 tumor suppressor protein. Stabilization and accumulation of wild-type p53 usually result in either cellular growth arrest or apoptosis. Since the HPV-16 E7 proteins can interfere with several cell cycle checkpoints and similar alterations in the levels of E2F-1, Rb and p53 are also observed in a p53-dependent response to DNA damage,[15,16,17] we investigated whether E7 expression alters this signal transduction pathway.

The purpose of this study is to investigate the effect of HPV-16 E7 genes in glioblastomas after irradiation under hypoxia. The differently expressed proteins and genes induced by HPV-16 E7 gene might be used to target proteins and genes to increase radiosensitivity during irradiation. Intuitively,

overexpression of HPV-16 E7 gene that affect the cell cycle at a specific check point should have a more profound inference on tumor suppression therapy.

II. MATERIALS AND METHODS

1. Cell Culture

The human glioma cell lines U87-MG cells with wild type p53. The cell lines were produced from American Type Culture Collection (ATCC). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM), (GIBCO BRL, Gaithersburg, USA) supplemented with 10% fetal bovine serum and Antibiotics (10mg/ml) (GIBCO BRL, Gaithersburg, USA). Cells were incubated at 37 C in 5% CO2 atmosphere.

2. Expression of HPV-16 E7 genes in glioma cells

Retroviral vector LXSN was used to express the HPV-16 E7 genes respectively, in addition to the neomycin-resistance gene. The construction and generation of the retroviruses has been described by Halbert and co-workers (1991). Controls included mock infected cells and cells infected with the same retroviral vector without the gene of interest, LXSN. Glioma cells expressing the viral genes were selected in 400mg/ml G418 (GIBCO BRL, Gaithersburg, USA) for 30 days. Stably proliferating cells were screened for E7 mRNA expression by reverse transcription (RT) poly-merase

chain reaction (PCR)

3. Hypoxia treatment and γ-Irradiation

To assess the effect of a low-oxygen environment on cell death, cells were incubated in an anaerobic culture chamber (Forma Scientific, OH, USA), washed three times with deoxygenated, glucose-free balanced salt solution (BSS0) containing 116mM NaCl, 1.8mM $CaCl_2$, 0.8mM $MgSO_4$, 5.4mM KCl, 1mM NaH_2PO_4, 14.7mM $NaHCO_3$, 10mM HEPES, 2% FBS and 10mg/L phenol red at pH 7.4 and incubated in BSS0 at 0% oxygen for 6h. So it would be possible to determine the effect by ionizing radiation on cell death. Cell were irradiated with 10Gy using a Gammacell 3000 Elan.

4. Flow cytometry analysis of cell death

Apoptosis was determined using an apoptosis detection kit (BioVision Inc., CA. USA). Briefly, cells were collected after treatment, washed twice in ice-cold PBS, and then resuspended in binding buffer at a density of 1×105 cells/mL. Cells were incubated simultaneously with FITC-labeled annexin V and propidium iodide (PI) for 10min. Flow cytometry was performed to determine the populations positive for annexin V-FITC (FL-1 channel) and/or PI (FL-2 channel) by a FACScan (Becton Dickinson, Mountain View, CA). Data were analyzed using Cell Quest software (Beckton Dickinson Immunocytometry Systems).

5. Two-dimensional electrophoresis.

Protein sample preparation : Cultured cell pellets were washed twice in

ice-cold PBS(GIBCO) and lysed in sample buffer composed with 7M urea, 2M Thiourea containing 4%(w/v) 3-[(3-cholamidopropy)dimethyammonio]-1-propanesulfonate(CHAPS), 1%(w/v) dithiothreitol(DTT) and 2%(v/v) pharmalyte and 1mM benzamidine. Proteins were extracted for one hour at room temperature with vortexing. After centrifugation at 15,000xg for one hour at 15℃, insoluble material was discarded and soluble fraction was used for two-dimensional gel electrophoresis. Protein loading was normalized by Bradford assay.

A. 2D PAGE

IPG dry strips were equilibrated for 12-16 hours with 7M urea, 2M thiourea containing 2% 3-[(3-cholamidopropy)dimethyammonio]-1-propanesulfonate(CHAPS), 1% dithiothreitol(DTT), 1% pharmalyte and respectively loaded with 200ug of sample. Isoelectric focusing(IEF) was performed at 20℃ using a Multiphor II electrophoresis unit and EPS 3500 XL power supply(Amersham Biosciences, Uppsala, Sweden) following manufacturer's instruction. For IEF, the voltage was linearly increased from 150 to 3,500V during 3 hours for sample entry followed by constant 3,500V, with focusing complete after 96 kVh. Prior to the second dimension, strips were incubated for 10 minutes in equilibration buffer(50mM Tris-Cl, pH6.8 containing 6M urea, 2% SDS and 30% glycerol), first with 1% DTT and second with 2.5% iodoacetamide. Equilibrated strips were inserted onto SDS-PAGE gels(20-24cm, 10-16%). SDS-PAGE was performed using Hoefer DALT 2D system(Amersham Biosciences, Uppsala, Sweden) following manufacturer's instruction. 2D gels were run at 20℃ for 1.7kVh. And then 2D gels were silver stained as described by Oakley et. al(Anal. Biochem. 1980, 105:361-363) but fixing and sensitization step with

glutaraldehyde was omitted.

B. Image analysis

Quantitative analysis of digitized images was carried out using the PDQuest software(version 7.0, BioRad) according to the protocols provided by the manufacturer. Quantity of each spot was normalized by total valid spot intensity. Protein spots were selected for the significant expression variation deviated over two fold in its expression level compared with control or normal sample.

C. Maldi-tof analysis

Enzymatic digestion of protein in-gel Protein spots were enzymatically digested in-gel in a manner similar to that previously described by Shevchenko et al(Anal. chem. 1996, 68:850-858) and using modified porcine trypsin. Gel pieces were washed with 50% acetonitrile to remove SDS, salt and stain, dried to remove solvent and then rehydrated with trypsin(8-10ng/ $\mu\ell$) and incubated 8-10h at 37℃. The proteolytic reaction was terminated by addition of 5$\mu\ell$ 0.5% trifluoroacetic acid. Tryptic peptides were recovered by combining the aqueous phase from several extraction of gel pieces with 50% aqueous acetonitrile. After concentration the peptide mixture was desalted using C18ZipTips(Millipore, Bedford, MA), and peptides eluted in 1-5$\mu\ell$ of acetonitrile. An aliquot of this solution was mixed with an equal volume of a saturated solution of -cyano-4-hydroxycinnamic acid in 50% aqueous acetonitrile, and 1$\mu\ell$ of mixture spotted onto a target plate, and then protein analysis were performed using a Ettan MALDI-TOF (Amersham Biosciences,

Uppsala, Sweden).

6. RNA preparation

The Total RNA was extracted from the cultured HUVEC using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The quantity of the total RNA was determined by an O.D. 260 reading at a spectrophotometer, GeneSpec III (Hitachi, Tokyo, Japan), and the quality was assessed by electrophoresis on an agarose gel and also by an assay on a Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA, USA). The Yonsei reference RNA (Cancer Metastasis Research Center, Seoul, Korea) was prepared by pooling the equivalent amounts of the total RNA from U87 MG (glioblastoma) cells and U87△E7 cells.

A. RNA amplification

The amplification of the total RNA was performed based on a previously described protocol 10 with a slight modification.

B. First strand cDNA synthesis

Two micrograms of the total RNA was used in the first round of amplification. The RNA template was mixed with 2㎍ of oligo-dT/T7 primer (5'-GGCCAGTGAATTGTAATACGACTCACTATAGGGAGGCGG-3', Genotech, Daejun, Korea) in total 9㎕ of RNase-free water. The RNA/primer mixture was denatured at 65℃ for 10 min and chilled on ice for 5 min. Four micro liters of the 5X first strand buffer (Invitrogen, Carlsbad, CA,

USA), 2㎕ of 100mM DTT, 2㎕ of 10mM dNTP mix (Invitrogen, Carlsbad, CA, USA), 2㎕ of RNAsin (Promega, Madison, WI, USA) and 2㎕ of SuperScript II (Invitrogen, Carlsbad, CA, USA) were added to the RNA/primer mixture and the reverse transcription reaction was performed at 42℃ for 1 hour.

C. Second strand cDNA synthesis

For the second strand cDNA synthesis, 91㎕ of RNase-free water, 30㎕ of 5X second strand buffer, 3㎕ of 10mM dNTP mix, 10U of DNA ligase, 4U of DNA polymerase I and 2U of RNase H were added to the finished first strand cDNA synthesis reaction mixture. The resulting solution was incubated at 16℃ for 2 h. T4 DNA polymerase was then added and incubated at 16℃ for 5 min. The reaction was stopped by adding 10㎕ each of 1M NaOH and 0.5M EDTA and incubated at 65℃ for 10 min, which was then followed by neutralization with the addition of 25㎕ of Tris-HCl (pH 7.5). The double stranded cDNA was extracted with an equal volume of phenol : chloroform : isoamyl alcohol (25:24:1) and precipitated with ethanol in the presence of 1㎕ Linear Acrylamide (0.1㎍/㎕, Ambion, Austin, Tx, USA). The dried pellet was re-suspended in 9㎕ of RNase-free water.

D. In vitro transcription

The mRNA was transcribed from the double-stranded cDNA using a T7 MEGAscript kit (Ambion, Austin, TX, USA). Briefly, 2㎕ each of 75mM NTP, enzyme mix and 10X reaction buffer was added to 8㎕ of the double stranded cDNA and the reaction mixture was incubated at 37℃ for 5 h. The

amplified mRNA was cleaned up using an RNeasy mini kit (Qiagen, Valencia, CA, USA) following the manufacturer's instructions. The quantity of amplified RNA was determined by an O.D.260 reading at a spectrophotometer. The integrity of the amplified mRNA was assessed by electrophoresis on an agarose gel and also by a Bioanaylzer 2100.

7. cDNA microarray

The cDNA microarray used a human cDNA chip (CMRC-GT, Seoul, Korea) containing 17,664 known genes and ESTs in a reference design. The test samples were labeled with Cy5 and were individually co-hybridized with the Cy3-labeled reference RNA. The Yonsei reference RNA (CMRC, Seoul, Korea) was made from 11 human cancer cell lines of various human organs.

A. Probe labeling and hybridization

Two micrograms of the amplified mRNA was labeled with Cy3- or Cy5-dUTP during reverse transcription. The RNA was first mixed with 6㎍ of the random primer (Invitrogen, Carlsbad, CA, USA) and incubated at 65℃ for 10 min. Eight micro liters of the 5X first strand buffer, 4㎕ of 100mM DTT, 2 ㎕ of SuperScript II RT, 2㎕ of 20X low-dT/dNTP mix, and 1㎕ of RNAsin were added to the RNA /random primer mixture and incubated at 42℃ for 2 h. The residual RNA was hydrolyzed by incubation at 65℃ for 30 min in the presence of 15㎕ of 0.1M NaOH and the reaction was neutralized with 5 ㎕ of HCl. The Cy3 and Cy5 labeled probes were purified using QIAquick PCR Purification Kit (Qiagen, Valencia, CA, USA). The purified probes were combined and mixed with 20㎍ Human COT-1 DNA (Invitrogen, Carlsbad,

CA, USA), 20㎍ yeast tRNA (Invitrogen, Carlsbad, CA, USA), and 20㎍ poly(A) RNA (Sigma, Saint Louis, MO, USA). The final probe wasconcentrated to 80㎕ using a Microcon YM-30 column (Millipore, Bedford, MA, USA) and denatured at 100℃ for 2 min. The cDNA microarrays were pre-hybridized in 3.5X Sodium chloride/Sodium Citrate buffer (SSC), 0.1% Sodium Dodesyl Sulfate (SDS), and 10㎎/㎖ Bovine Serum Albumin (BSA) at 42℃ for 1 h prior to probe application. The probe was hybridized in 25% formamide, 5X SSC and 0.1% SDS at 42℃ for 16 h. Following hybridization, the arrays were washed in 2X SSC with 0.1% SDS, 1X SSC with 0.1% SDS, 0.2X SSC, and 0.05X SSC, sequentially washed for 2 min each and then spun dried at 500x g.

B. Image scanning and data processing

The fluorescence signals on the microarrays were acquired using a GenePix 4000B scanner (Axon Instruments, Foster City, CA, USA). The scanned images were processed using GenePix Pro 4.0 software (Axon Instruments, Foster City, CA, USA). A computation of the background intensity was automatically performed as the local background subtraction method in order to reduce the effect of the non-specific fluorescence. Systemic errors were corrected by normalization of the log2-transformed data using intensity dependent, within-print tip normalization based on the Lowess function14. After normalization, genes with more than 20% of the missing value were filtered.

8. Data analysis

The Pearson correlation coefficient among the triplicate microarrays was measured using S-Plus 2000 software (Insightful, Seattle, WA, USA).

Hierarchical clustering analysis was performed with Cluster software and the resulting dendrogram was visualized using TreeView software (http://rana.lbl.gov/EisenSoftware.htm). Cluster analysis was performed to organize the microarray data so that the underlying structures could be recognized and explored. A display of microarray data used the Box plot and M-A plot with S-Plus 2000. The box plot is a simple graphical summary of the log ratio distribution of the data set. This plot displays a statistical summary consisting of the median, upper, and lower qualities as well as the range. The central box in a plot represents the Inter Qualities Range (IQR), which is defined as the difference between the 75th and 25th percentile, or the upper and lower qualities. The M-A plot shows the ratio information rotated by 45 with the axes scaled. In the M-A plot, the log ratios (M=log2R/G) are plotted on the y-axis against the log the geometric mean of the signal strengths (A=log2 R .G) for each spot on the slides. This plot serves to represent the range the differential expression patterns. The selection of the genes differently expressed in any two groups was used a statistical program of Significance Analysis of Microarray (SAM)16. SAM uses an algorithm based on the Student t-test and also performs data permutations to determine the False Discovery Rate (FDR), which is the percentage of genes falsely reported as having statistically significant differential expression. The annotation of the selected genes was performed using the Database for Annotation, Visualization and Integrated Discovery (DAVID) (http://apps1.niaid.nih.gov/david) and Stanford Online Universal Resource for Clones and Expressed sequence tags (SOURCE)(http://source.stanford.edu/cgi-bin/source/sourceSearch)

9. Western blot analysis

Equal amounts of protein were subjected to electrophoresis on 10% SDS-polyacrylamide gels. Separated proteins were then electro-transferred to Immobilion-NC membrane (Millipore, Bedford, MA). The membranes were blocked for 1h at room temperature in 5% skim milk in TBS plus 0.1% Tween-20 (TBS-T). The membranes were incubated overnight with anti-Bcl2, anti-Hsp70, anti-CDC2, anti-Rb, anti-Bax, anti-p53, and anti-VEGF. After washing 3 times with TBS-T for every 5min, blots were incubated with peroxidase-conjugated anti-rabbit or anti-mouse secondary antibodies for 1h at RT. Finally, blots were rinsed and proteins were visualized using an ECL protein detection kit according to the manufacturer's instructions.

10. Semi-quantitative RT-PCR

We performed semi-quantitative RT-PCR experiments to verify the microarray data. To adjust for variable quantities of cDNA among samples, we quantified expression of an internal control gene, 18sRNA, for which the designed primers were 5'-CGGCTACCACATCCAAGGAA 3' (forward) and 5'-GCTGGAATTACCGCGGCT-3' (reverse). After adjusting for the variations by dilution of the samples, semi-quantitative RT-PCR was performed for each of six selected genes, p130, p107 , Rb1, Il-16, IL-13 Primers were designed using the Primer 3 program (The Whitehead Institute, Boston, MA), and their specificity was tested by reference to a sequence database (National Library of Medicine, Bethesda, MD) PCR was carried out using the GeneAmp PCR

system 9700 (Applied Biosystems, Foster City, CA), initiated with 94C denaturing for 5 min, followed by 25.35 cycles of 94C for 30 s, 60C for 30 s and 72C for 30 s, and final extension for 30 min at 72C. PCR products were detected by scanning a 2% agarose gel stained with ethidium bromide after electrophoresis, using a digital imaging system (AlphaImager 3300; Alpha Innotech, San Leandro, CA). The spot-density method was applied according to the manufacturer's instructions. All the experiments were duplicated. To acquire an objective index of the expression level for each gene, the density score from the PCR-product band of each gene of interest was divided by that of b-actin, and was designated as the "expression ratio". Statistically significant differences between expression ratios of normal tissues versus GBM tissues were analyzed by Student's t-tests; P values smaller than 0.05 were considered significant.

11. Statistical analysis

Results are presented as the mean ± SEM of at least three different experiments performed in separate cell preparation, duplicate or triplicate determination were performed in each experiments. One-way ANOVA followed by Student's t-test was used as indicated in order to examine the statistical significance; p-values less than 0.05 were considered significant.

III. RESULTS

1. Retroviral infection of HPV-16 E7 gene

The HPV-16 E7 genes infected U87 MG cells represented 300bp cDNA expression after gene delivery (Fig. 1A). Primer design for detection of HPV-16 E7 genes and cDNA expression of U87 MG cells after infected with HPV-16 E7 genes (Fig. 1B).

2. Cell death induced by HPV-16 E7 gene in U87-MG cells after irradiation under hypoxia

Cells were stained with both PI and FITC-AV, then analyzed by flow cytometry. Necrotic cells are demonstrated by AV^-/PI^+ staining, since PI enters cells when membrane integrity is lost and binds nucleic acids. Apoptotic cells are demonstrated by AV^+/PI^- staining, since annexin V binds to phosphatidylserine that translocates to the outer side of the plasma membrane during apoptosis. AV^+/PI^+ stained cells are likely to be late apoptotic or necrotic and AV^-/PI^- cells represent viable cells. First of all, cell death is generally increased in U87△E7 rather than U87 cells (Fig. 2). In hypoxia condition cell death is little different either transfected cells or nontransfected cells. but In hy4+10gy condition resulted in a significant increase in the percentage of cell death from 17.37% to 25.37%. In 10gy condition found out the increase of cell death U87△E7 (19.6%) compared to U87-MG (10.5%). These results indicate that E7 gene caused increasing the radiosensitivity during the irradiation.

3. **Differently expressed proteins in u87-MG cells and u87△E7 cells.**

Proteomic studies to date, other than in biomedicine, have focused mainly on changes in genome expression that are triggered by environmental factors. Proteomic analysis is required to determine which proteins have been conditionally expressed and whether any post-translational modifications are affected. Protein profile changes in response to specific E7 genes can provide useful marker proteins that was related to cell death. As shown in Fig. 3, we could clearly separate the up-regulated and down-regulated proteins, ranging from low to high molecular weights on the 2-D gel. Most proteins were separated in the isoelectric point range of 4-7. Proteins extracted from untreated control were used as the control for comparative analysis. About 113 protein spots were detected in control with computer-aided image analysis of 2-D gel. Transfected of HPV-16 E7 gene resulted in an increase in the net synthesis of some proteins and a decrease in the net synthesis of others. Out of 380 protein spots detected on 2-D PAGE, 11 spots were differently expressed in response to E7 gene when stained with Coomassie Brilliant Blue. Overall protein expression profiles of three replicates of each treatment were identical, indicative of high reproducibility. Among the differently expressed spots, we identified three spots(tumor necrosis factor ligand superfamily, member 11 , Phosphoglycerate mutase 1, TATA binding protein) (Fig. 4).

4. Differently expressed genes by microarray

We examined genome-wide gene-expression profiles of U87 and U87△E7 using a cDNA microarray consisting 17,114 human genes. We observed a significant difference in the expression level of 271 genes between U87-MG cells and U87△

E7 cells. Among the 271 genes, 32 were up-regulated (P<0.000001 with at least a 3.0-fold difference) and 30 were down-regulated (P<0.00001 with at least a 2.0-fold difference) in their expression levels (Fig. 5). The 32 genes that were commonly up-regulated in U87△E7 cells and 30 that were commonly down-regulated are listed in the Table 1, 2. Many of the genes with increased expression in U87△E7 cells encode proteins that are involved in cell cycle or that have been reported to affect cell proliferation. Almost of the genes with decreased expression were stress response proteins that have been reported to affect protein modification and cell survival.

5. Effect of HPV-16 E7 gene on the expression of anti-apoptotic and apoptotic proteins in U87-MG cells after irradiation under hypoxia.

Following above results, HPV-16 E7 gene induced cell death through the regulating cell cycle and anti-apoptotic proteins. To confirm these data, we performed the western blot analysis of anti-apoptotic proteins, Bcl-2 and Hsp70. The expression of Bcl-2 is generally decreased in U87△E7 cells rather than U87-MG cells (Fig. 6). In case of Hsp70, the expression level is significantly decreased at hypoxia condition, hy6+10gy and hy6 (Fig. 6). However HPV-16 E7 gene didn't influence in irradiation condition. These results demonstrated that as the E7 gene regulate the anti-apoptotic proteins, influenced cell death. Since p53 was known to a key modulator of cellular stress responses, and the activation of p53 can trigger apoptosis. also p53 can mediate apoptosis by inducing the expression of Bax. We investigated the role of HPV-16 E7 gene in the expression p53 and Bax levels after irradiation under hypoxia. The expression level of p53 and Bax by western

blot analysis (Fig. 6). In normoxic cells, p53 and Bax were not mainly expressed. In transfected cells, however, the expression of p53 were significantly increased regardless of hypoxia and radiation condition. The expression level of Bax is increased generally in transfected cell. These data suggest that HPV-16 E7 gene may be involved with expression of p53 and Bax.

6. The expression of cell cycle regulator protein by HPV-16 E7 gene.

E2F is the cell cycle-regulatory transcription factor, activate the cell cycle regulatory gene. Phosphorylation of pRB by Cdk2, Cdk4, and Cdk6 results in release and activation of E2F, which induces transcription of genes essential for late G1 progression and S phase entry. The expression of E2F-1and cdc2 were increased in U87△E7 cells at hypoxic condition, but the expression of E2F-1 and were observed to decrease in U87△E7 when cells were exposed to irradiation after hypoxia. we could find out mostly increase of E2F expression in U87△E7 cells rather than U87-MG cells(Fig. 7). These data suggest that HPV-16 E7 gene may be related to the expression of E2f-1 and cdc2.

7. The mRNA expression of Rb family. RT-PCR analysis of Rb, p130 and p107 after irradiation under hypoxia. One of the Rb family ,the expression of p130 is little decreased in U87△E7 cells. but Rb and p107 is no diffenence(Fig. 8). The expression of p130 and p107 bind E2F4or E2F5, which is repressor E2F, cause the cell cycle arrest in G1/S[39]. Therefore increase of radiosensitivity by HPV16 E7 gene may be associated with the G2/M phase arrest not the G1/S phase.

(A)

E7 primer

sense 5-ACCATGCATGGAGATACACCTAC-3

antisense 5-ATTCTTATGGTTTCTG-3.

(B)

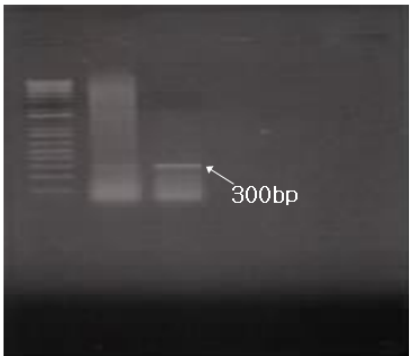

Figure 1. Infection of retroviral vector expressing HPV-16 E7 gene and identificaion. To confirm expression of the HPV-16 E7 gene in U87-MG cells infected with the retroviral vectors. (A) RT-PCR was carried out using the primer for HPV-16 E7 gene indicated. (B) The expected amplification products, 300bp fragment were found in U87△ E7 cells.

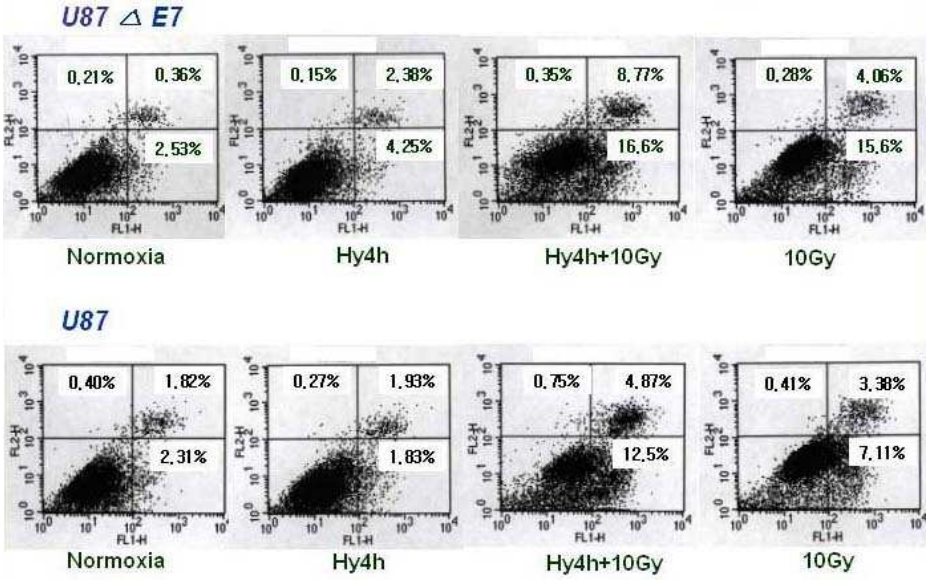

Figure 2. Representative dot plots of flow cytometry of cell death in U87△E7 cells and U87-MG cells after radiation under hypoxia. Cells were stained with propidium iodide (PI) and FITC-Annexin V (AV). Samples were analyzed by flow cytometry. The numbers above are percentage value. In each experiment, the data from 5,000 to 10,000 cells were collected and analyzed.

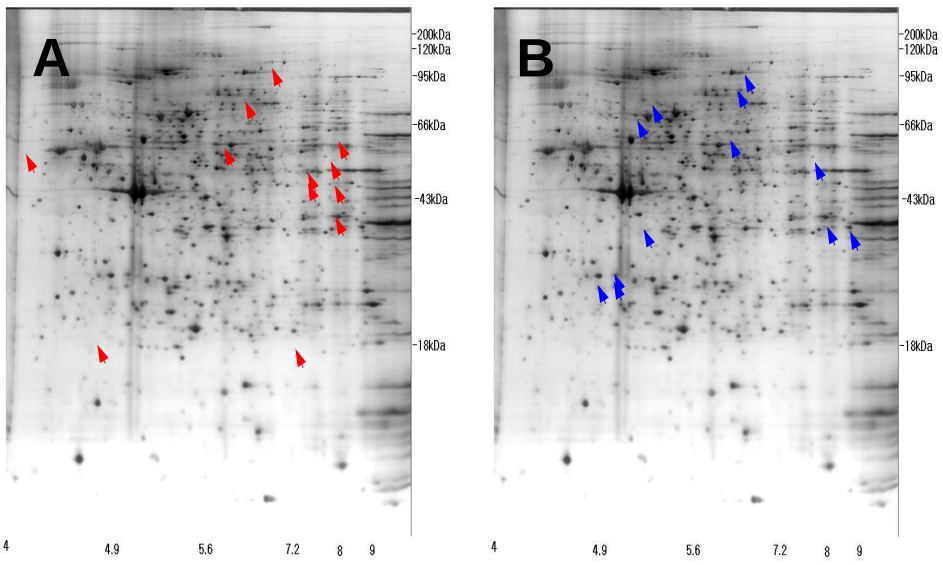

Figure 3. 2D protein expression maps of U87△E7 cells and U87-MG cells. 2D gels were generated, stained, and analyzed as described in text. Differently expressed proteins are depicted by arrows. **A,** red arrows show increased expression U87△E7 cells than U87-MG cells. **B,** blue arrows show decreased expression U87E7 than U87-MG cells.

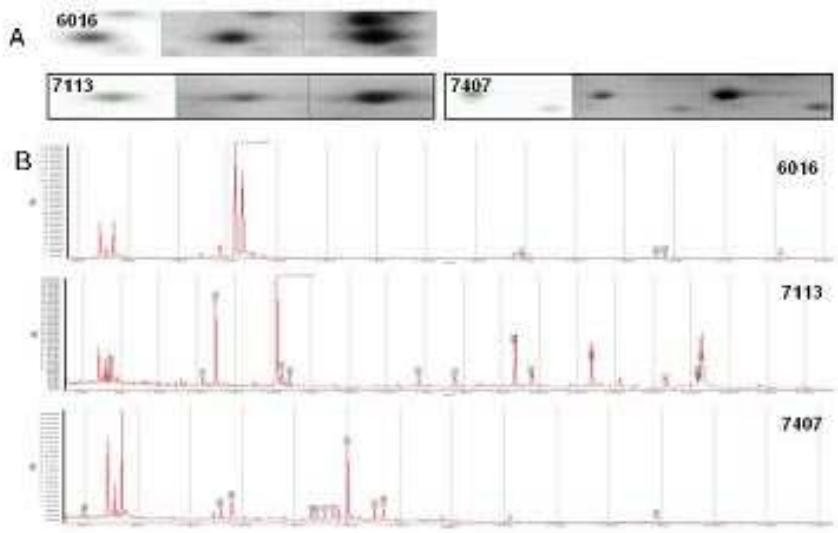

Figure 4. Identification of differently expressed protein in U87-MG cells and U87△E7 cells. A: 2D gel regions in U87MG (left) and U87△E7(right) of spots identified as 6016 : tumor necrosis factor ligand superfamily, member 11 , 7113 : Phosphoglycerate mutase 1, 7407 : TATA binding protein interacting protein in U87MG and U87△E7 cells. B: MS/MS analysis of gel spots.

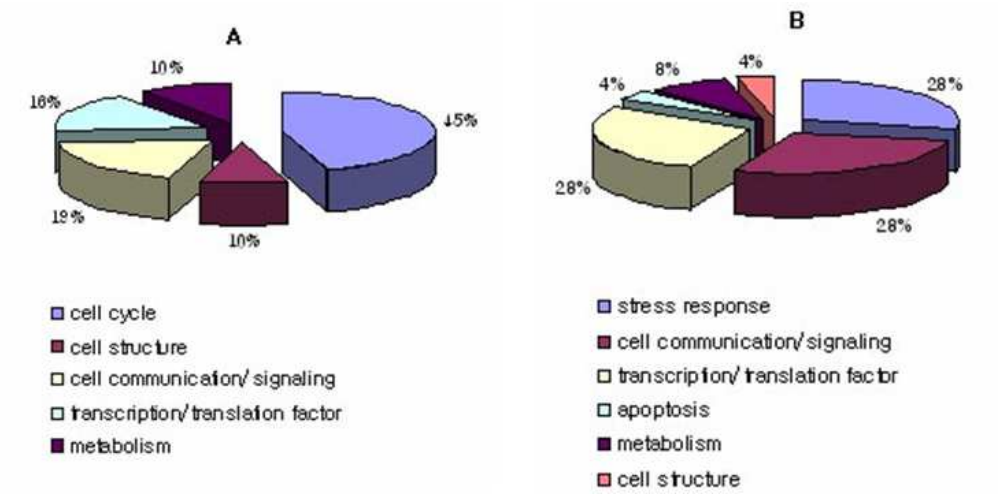

Figure 5. Functional annotation of 57genes specific to activated E7 gene from glioblastoma. (A) up-regulated 32 genes, (B) down-regulated 25 genes.

Table 1. Up regulated genes in U87MG Cells transfected with E7

Category Gene name		Symbol	Fold change
cell cycle			
cell division cycle 2, G1 to S and G2 to M		CDC2	20.8
pituitary tumor-transforming 1		PTTG1	22
cyclin B2		CCNB2	18.5
protein regulator of cytokinesis 1	PRC1	8.4	
MAD2 (mitotic arrest deficient, yeast, homolog)-like 1		MAD2L1	14.5
phorbolin (similar to apolipoprotein B mRNA editing protein)		APOBEC3B	3.68
serine/threonine kinase 15		STK6	3.69
cyclin B1		CCNB1	7.91
citron (rho-interacting, serine/threonine kinase 21)	CIT	2.83	
activator of S phase kinase		ASK	3.75
leukemia-associated phosphoprotein p18 (stathmin)	STMN1	3	
budding uninhibited by benzimidazoles 1 (yeast homolog)		BUB1	4.65
cell division cycle 25B		CDC25B	2.78
cell communication/signaling			
corticotropin releasing hormone		CRH	4.59
kinesin-like 5 (mitotic kinesin-like protein 1)		KIF23	5.91
karyopherin alpha 2 (RAG cohort 1, importin alpha 1)		KPNA2	3.52
hepatoma-derived growth factor (high-mobility group protein 1-like)		HDGF	3.59
diazepam binding inhibitor (GABA receptor modulator, acyl-Coenzyme A binding protein)		DBI	2.48
transcription/translation factor			
centromere protein A (17kD)		CENPA	7.56
centromere protein F (350/400kD, mitosin)		CENPF	7.46
Lsm3 protein		LSM3	2.76
early growth response 1		EGR1	3.4
BRCA1 associated RING domain 1		BARD1	2.97

Table 1. Down regulated genes in U87MG Cells transfected with E7

Category Gene name	Symbol	Fold change
Stress response		
heat shock 70kD protein 1B	HSPA1B	-6.49
heat shock 105kD	HSPH1	-5.17
DnaJ (Hsp40) homolog, subfamily B, member 1	DNAJB1	-2.72
gephyrin	GPHN	-6.29
protein kinase H11; small stress protein-like protein HSP22	HSPB8	-5.95
tumor rejection antigen (gp96) 1	TRA1	-2.15
DnaJ (Hsp40) homolog, subfamily B, member 9	DNAJB9	-2.49
Cell communication/signaling		
calmegin	CLGN	-3.3
tumor-associated calcium signal transducer 1	TACSTD1	-5.44
ras homolog gene family, member E	ARHE	-2.44
solute carrier family 3 (activators of dibasic and neutral amino acid transport), member 2	SLC3A2	-2.4
jagged 1 (Alagille syndrome)	JAG1	-2.65
immunoglobulin superfamily, member 4	IGSF4	-2.34
GRB2-associated binding protein 1	GAB1	-2.53
Transcpirtion/translation factor		
v-maf musculoaponeurotic fibrosarcoma (avian) oncogene homolog	MAF	-4.57
spleen focus forming virus (SFFV) proviral integration oncogene spi1	SPI1	-3.15
Down syndrome critical region gene 1	DSCR1	-3.9
runt-related transcription factor 1 (acute myeloid leukemia 1; aml1 oncogene)	RUNX1	-2.6
nuclear factor (erythroid-derived 2)-like 1	NFE2L1	-2.34
plasminogen activator, urokinase receptor	PLAUR	-2.91
Homo sapiens mRNA; cDNA DKFZp434K098 (from clone DKFZp434K098); partial cds	CNOT6L	-2.34
Apoptosis		
BCL2-associated athanogene 3	BAG3	-3.35

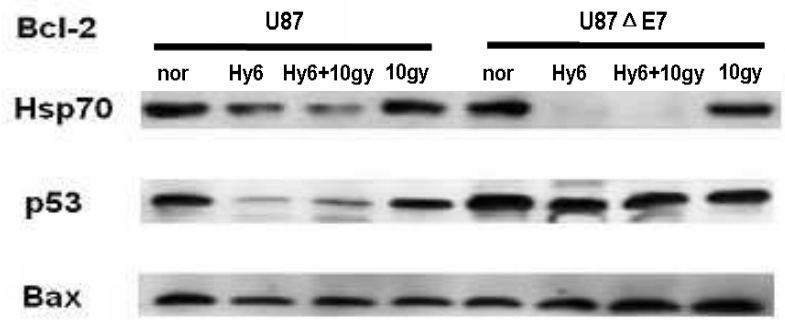

Figure 6. Effect of HPV-16 E7 gene on the expression of anti-apoptotic and apoptotic proteins. Western blot analysis of Bcl-2, Hsp70, p53 and Bax after irradiation under hypoxia. Cell lysates (40μg of protein) from experimental groups were analyzed by western blotting using antibodies against Bcl-2, Hsp70, p53 and Bax.

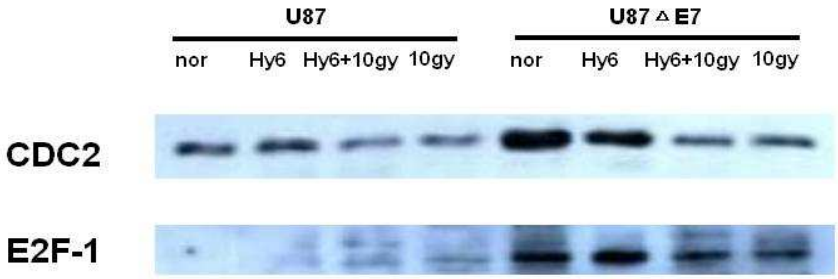

Figure 7. The expression of cell cycle regulator protein by HPV-16 E7 gene. Western blot analysis of cdc-2 and E2F-1 after irradiation under hypoxia. Cell lysates (40μg of protein) from experimental groups were analyzed by western blotting.

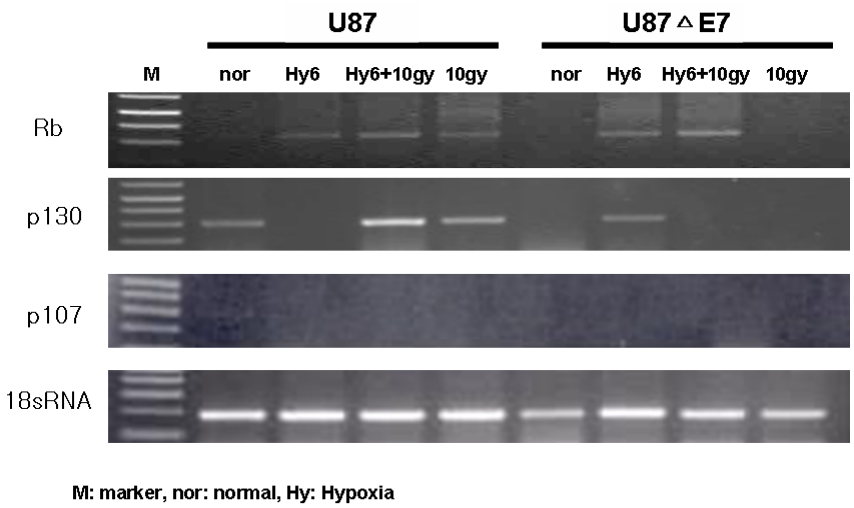

Figure 8. The mRNA expression of Rb family. RT-PCR analysis of Rb, p130 and p107 after irradiation under hypoxia. 18sRNA is a constitutive expressed gene. Cell lysates (2μg of total) from experimental groups were used

IV. DISCUSSION

Human solid tumors are less oxygenated than normal tissues. This leads to resistance to radiotherapy and anticancer chemotherapy[18,19]. U-87 MG cells with wild-type p53 were resistant to hypoxia and ionizing radiation as described in other many studies[3,5].Many of genes encoding proteins induced by hypoxia are potential products for modifying the radiation response of normal or malignant tissues.

The human papilloma virus-type 16 (HPV-16) E7 proteins that bind the retinoblastoma protein (Rb) and facilitate its dissociation from E2F induced apoptosis[20,21,22,23]. Selective degradation or inhibition of cellular proteins, such as p53 and pRb, with negative growth regulatory functions may affect cell viability and influence the likelihood of a cell undergoing apoptosis. HPV-16 E7 genes can be exploited in overcoming the resistance to radiotherapy for cancer treatment. In this study, we investigated the radiosensitivity of the proteins induced by radiation and hypoxia and the effect of HPV-16 E7 gene expression on radiosensitivity. When HPV-16 E7 gene was transferred into the cell, the susceptibility of apoptosis was increased by undergoing hypoxic treatment before radiation in the U87△E7 cells. The level of p53 was increased in the U87△E7 cells both hypoxia and irradiation. Also the expression of E2F-1 is increased in U87△E7 cells at hypoxic condition, but the expression of E2F-1 was observed to decrease in U87△E7 when cells were exposed under hypoxia after irradiation. we could find out mostly increase of E2F expression in U87△E7 cells rather than U87-MG cells. The level of E2F may contribute to some critical factors of cellular repair function associated with DNA damage, and to deciding whether the cells will pass through cell cycle arrest[24,25].

Level of p53 is important in radiation-mediated apoptosis of tumors[26,27]. Transfection of HPV16-E7 genes into the cancer cell line provides an additional mechanism for transformed cells to suffer the apoptosis and the susceptibility of radiation resistance. In this study, we investigated which proteins have been expressed differently in U87△E7 cells and we identified one spot, tumor necrosis factor ligand superfamily from 115 protein spots by 2-dimensional electrophoresis. The Cytokines Tumor Necrosis Factor-α (TNF-α) and TNF-related Apoptosis-inducing Ligand differently modulate proliferation and apoptotic pathways in human keratinocytes expressing the human papilloma virus-16 E7 oncoprotein[28]. It is reported that TNF family associated with cell death and inflammation[29]. Thus HPV16 E7 gene may be involved with cell death through the regulating TNF family. The level of examination of the gene, a study of cDNA microarray revealed the genes to increases of 159, and the genes taken part of the cell cycle were observed about 14 of them in U87△E7 cells. Especially CDC2, cyclin B2, PTTG1 and MAD2L1 is the control of Cell period of G2/M phase, the degree of their gene expression increased about ten-times. Catalytic subunit of CDC2 MPF(M-Phase promoting factor)[30,31], transition of G1/S, G2/M is major part of cell cycle. PTTG1 contributes on stabilization of chromosomes in P53 pathway[32,33]. Also, it is known as primary resources on the repair of DNA. The MAD2L1, stops the conversion between metaphase and anaphase until the arrange correctly from M phase[34,35]. The exception of cell structure, cell communication/signaling, transcription/translation factor, metabolism, other gene expression were not found. The present HPV-16 E7 oncogene, adjust the cell cycle, therefore it gives rise to sensitive generation. Also, the reduction of differently expressed

genes were 111 and mainly were related to stress response which are heat shock 70kD protein 1B, heat shock 105kD {DnaJ (Hsp40) homolog, subfamily B, member 1}, DnaJ {(Hsp40) homolog subfamily B, member 9}, tumor rejection antigen (gp96) 1, the HSP family, known as the stabilization and protection of protein[36]. Besides that, Bcl2-associated athanogene 3 is the gene that participates in anti-apoptosis. In this study, the expression of Bcl-2 and Hsp70 were decreased in U87△E7 cells rather than U87-MG cells under hypoxic condition. It is reported that exposure to hypoxic stress for three weeks does not change Hsp70 levels in total lung tissue, but a significant reduction of Hsp70 expression occurs in bronchiolar epithelial cells[37].

One of the Rb family, the expression of p130 was also little decreased in U87△E7 cells, but Rb and p107 was no diffenence. In G0 and early G1, p107 and p130 form repressor complexes in conjunction with E2F4 or E2F5 at most if not all E2F-responsive promoters[38]. The expression of p130 and p107 bind E2F4 or E2F5, which is repressor E2F, cause the cell cycle arrest in G1/S[39]. As shown at Table 2, cdc2 and cyclin B1 were significantly down regulated in U87△E7 cells. G_2/M transition is strictly controlled by the complex kinase cyclin B1/cdc2, also known as the mitosis promoting factor (MPF). During the cell cycle, cyclin B1 accumulates in G_2/M[40]. Another mechanism is important in the regulation of the cell cycle entry into mitosis, the subcellular localization of cdc2 and cyclin B1, which is suddenly imported into the nucleus at the end of G_2[41]. Also MAD2 was down regulated in U87△E7 cells. MAD2 has been demonstrated to be an essential gene even in tumor cells such that near complete elimination of this protein from cancer cells results in p53 independent cell death[42]. Therefore increase of radiosensitivity by HPV16 E7

gene may be associated with the G2/M phase arrest not the G1/S phase. Here we demonstrated that the anti-apoptotic genes and HSP family genes were decreased and the regulating genes of cell cycle G2/M phase were increased in the U87△E7 cells after irradiation under hypoxia. These results might suggest that the expression of HPV-16 E7 oncogene in U87-MG cells can induce the vulnerability of the cells to radiosensitivity.

V. CONCLUSION

This present study showed increases of proteins and genes related to radiosensitivity induced by HPV16 E7gene. These results have demonstrated following conclusions.

1. HPV16 E7 gene causes increasing the radiosensitivity during the irradiation under hypoxia in U87-MG cell.
2. The expression of TNF family was increased in U87△E7 cell after irradiation under hypoxia.
3. HPV16 E7 gene induce the decrease of anti-apoptotic proteins, HSP70 and Bcl-2, but the apoptotic proteins, p53 and Bax were increased by HPV16 E7 gene.
4. HPV16 E7 gene causes cell death via regulating genes of cell cycle G2/M phase after irradiation under hypoxia.

These results might suggest that the expression of HPV-16 E7 oncogene in U87-MG cells can induce the vulnerability of the cells to radiotherapy.

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Abstract (in korean)

저산소하에서 방사선 조사후 형질전환된 U87 세포의 프로테옴과 마이크로에레이 분석.

<지도교수 이 종 은>

연세대학교 대학원 의과학과

김 한 조

고형종양의 미세환경에서 반드시 존재하게 되는 저산소분획(hypoxia)은 암세포의 방사선 항암 요법의 결정적 장애가 되므로 방사선 항암 요법의 효과를 증진 시키기 위한 암세포의 선택적 치료기술에 대해 연구되고 있다. 최근 세포 증식에 있어서 종양억제 유전자와 그들의 역할에 관하여 관심이 집중되어 종양 억제 유전자나, 세포주기 조절 유전자들의 조절 기전에 관한 연구 역시 항암 요법 개발에 요구되고 있다. 사람 자궁암 유발 종양 유전자인 HPV type 16의 E6와 E7 단백질의 경우, 이들은 종양억제단백질 중 하나인 p53이나 pRB와 연관되어 있으며, 특히 이들 조절 인자들의 작용은 Rb의 기능과 연관되어 연구가 활발하게 진행되고 있다. pRb를 막는 인간 자궁암 바이러스의 type16 E7유전자는 DNA를 손상시키는 물질에 의한 세포성장 멈춤을 막을 수 있다.

이번 연구에서는 저산소하에서 방사선 조사후, U87과 U87ΔE7에서 다양한

단백질과 유전자의 발현차이를 볼 수 있었다. 우리는 2-D시스템을 통해 MALDI분석한 결과, 세포 사멸과 관련된TNF family를 포함한 발현의 변화를 보인 171개의 현저히 차이 나는 점들을 관찰하였고, 또한 Microarray를 통해 과발현된 159개의 유전자와, 저발현된 111개의 유전자를 볼 수 있었다. 과발현된 유전자는 세포사멸과 주로 세포주기의 G2기의 정지와 관련되어있었고, 저발현된 유전자는 세포생존과 스트레스 반응에 관여하는 유전자들이었다. 그러므로 E7유전자는 저산소하에서 방사선 조사후 E7유전자가 도입된 암세포인 U87△E7 세포에서 G2기 정지에 관련된 유전자들을 과발현시키고, 세포생존에 관여하는 Hsp70나 BCl2 단백질의 발현은 감소시켰으며, pro-apoptotic 단백질인 Bax와 p53의 발현을 유도함으로써 세포사멸을 유도한다고 생각된다.

핵심되는 말 : HPV16 E7 gene, 저산소분획, 방사선, 신경종양, 세포주기