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Identification of Differentially Expressed Radiation-induced Genes in Cervix Carcinoma Cells Using Suppression Subtractive Hybridization

Jun-Sang Kim, M.D.*§, Young-Sook Lee, M.S.§, Jeung Hoon Lee, M.D.†, Woong-Hee Lee, M.S.‡, Eun Young Seo, M.S.† and Moon-June Cho, M.D.*§

Departments of *Radiation Oncology, †Dermatology, College of Medicine, ‡Biology, College of Natural Sciences, §Cancer Research Institute, College of Medicine, Chungnam National University, Daejeon, Korea

Purpose: A number of genes and their products are induced early or late following exposure of cells to ionizing radiation. These radiation-induced genes have various effects on irradiated cells and tissues. Suppression subtractive hybridization (SSH) based on PCR was used to identify the differentially expressed genes by radiation in cervix carcinoma cells.

Materials and Methods: Total RNA and poly $(A)^+$ mRNA were isolated from irradiated and non-irradiated HeLa cells. Forward- and reverse-subtracted cDNA libraries were constructed using SSH. Eighty-eight clones of each were used to randomly select differentially expressed genes using reverse Northern blotting (dot blot analysis). Northern blotting was used to verify the screened genes.

Results: Of the 176 clones, 10 genes in the forward-subtracted library and 9 genes in the reverse-subtracted library were identified as differentially expressed radiation-induced genes by PCR-select differential screening. Three clones from the forward-subtracted library were confirmed by Northern blotting, and showed increased expression in a dose-dependent manner, including a telomerase catalytic subunit and sodium channel-like protein gene, and an ESTs (expressed sequence tags) gene.

Conclusion: We identified differentially expressed radiation-induced genes with low-abundance genes with SSH, but further characterization of theses genes are necessary to clarify the biological functions of them.

Key Words: Cervix carcinoma, Suppression subtractive hybridization, HeLa cells, Radiation-induced genes

Introduction

Carcinoma of the uterine cervix is a main cause of cancer death in women worldwide, especially in underdeveloped countries.[1,2] Although a number of factors influence the choice of treatment for early or locally advanced cervical carcinoma, the main treatment modalities are radiation therapy and surgery. Radiation therapy is an important treatment for early to advanced cervical carcinoma with either curative or palliative aims. To further improve local tumor control in uterine cervix carcinoma it is necessary to clarify the molecular mechanisms involved, including the mechanisms of radiation-induced tumor cell death, apoptosis, and signaling pathways.

A number of genes and their products are induced early or late following the exposure of cells to ionizing radiation. These include transcription regulating genes, such as c-jun, c-fos, and early growth response-1 (EGR-1),[3,4] cell-cycle-related genes, such as gadd45,[5,6] acute inflammatory response cytokines and growth factors, such as tumor necrosis factor (TNF), interleukin-1 (IL-1), and epidermal growth factor receptor (EGFR),[7,8] and apoptosis-related genes, such as p53.[9] These radiation- induced genes have various effects on cells and tissues after irradiation.

Suppression subtractive hybridization (SSH) is a powerful method for identifying tissue-specific and low-abundance transcripts. This technique can enrich rare differentially expressed cDNA sequences over 1,000-fold in a single round of sub-

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Reprint request to Moon-June Cho, Department of Radiation Oncology, Chungnam National University Hospital, 640 Daesa-dong, Jung-gu, Daejeon 301-721, South Korea
Tel: 042)220-7861, Fax: 042)220-7899
E-mail: mjcho@cnu.ac.kr
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tractive hybridization.[10,11] The HeLa cell was the first continuously cultured strain isolated from a human uterine cervical carcinoma, and it has been used in much biomedical research. We used the HeLa cell line as a model of uterine cervix carcinoma in this study. We used SSH to identify genes differentially expressed by radiation in the HeLa cell line compared with non-irradiated HeLa cells, to clarify the effect of radiation on uterine cervical carcinoma.

Materials and Methods

1. Cell line and irradiation

The human cervical carcinoma cell line, HeLa, was maintained in culture in Dulbecco's Modified Eagle's Medium supplemented with 100 units/ml penicillin and 100μg/ml streptomycin, and 10% fetal bovine serum. For the experiments, HeLa cells were irradiated with a single dose of 10 Gy at room temperature using a linear accelerator at a dose rate of 2 Gy/min. Before irradiation, the cells were washed twice with phosphate-buffered saline (PBS), which was also added to the cell cultures during irradiation. After irradiation, the cells were washed twice with PBS, and fresh culture medium was added to the dishes. The HeLa cells were harvested after a 4 h incubation period.

2. Isolation of total RNA and poly $(A)^+$ mRNA

Total RNA was isolated 1 h after irradiating HeLa cells using the SV total RNA isolation system (Promega, Madison, WI). Total RNA was mixed with 1×DNase I and buffer at 37°C for 30 min to decontaminate the genomic DNA. The mRNA was isolated using the PolyATtract mRNA isolation system (Promega, Madison, WI). The total RNA samples were quantified by measuring the absorbance at 260 nm using a spectrophotometer and the 28S and 18S bands were confirmed by agarose gel electrophoresis.

3. Suppression subtraction hybridization (SSH)

SSH was performed with a Clontech PCR-Select cDNA Subtraction Kit (Clontech Laboratories Inc., Palo Alto, CA). In brief, SSH was performed by synthesis of cDNA from the mRNA of testers (irradiated cells with 10 Gy) and drivers (non-irradiated cells). The cDNA was treated with restriction enzymes to make shorter and blunt-ended molecules. Two tester populations are created with different adaptors, but driver cDNA has no adaptor. Through first hybridization, differentially expressed genes were equalized and enriched. Then templates for PCR amplification are generated from differentially expressed sequences through second hybridization. Finally differentially expressed genes are amplified by performing of two cycles of PCR.

In the forward subtraction, irradiated HeLa cells were used as the tester and non-irradiated HeLa cells as the driver, and vice versa for the reverse subtraction. Driver cDNA was made from 2μg of poly $(A)^+$ RNA by oligonucleotide priming and using of T4 DNA polymerase according to the manufacturer's protocol. The resulting driver cDNA pellet was digested with *Rsa*I (37°C, 3 h) to produce blunt-ended cDNA synthesis. The cDNA was then phenol-extracted, ethanol-precipitated, and suspended in 7μl of deionized water. RsaI-digested tester cDNA was prepared as described for the driver cDNA. Two microliters of diluted tester cDNA were ligated to 2μl of adaptor 1 and adaptor 2 (10μM) in separate ligation reactions in a total volume of 10μl at 16°C overnight, using 0.5 units of T4 DNA ligase in the manufacturer's buffer. After ligation, 1μl of 0.2 M EDTA was added and the samples were heated at 70°C for 5 min to inactivate the ligase and stored at -20°C.

Two microliters of driver cDNA were added to each of two tubes containing 2μl of adapter 1- and adapter 2-ligated tester cDNA. The samples were mixed, ethanol precipitated, and then resuspended in 1μl of hybridization buffer. The solution was overlaid with mineral oil, and the DNA was denatured (1.5 min, 98°C) and then allowed to anneal for 10 h at 68°C. After this first hybridization, the two samples were combined and a fresh portion of heat-denatured driver in 1.5μl of hybridization buffer was added. The sample was allowed to hybridize for an additional 10 h at 68°C. The final hybridization was then diluted in 200μl of dilution buffer, heated at 72°C for 7 min, and stored at -20°C.

We amplified the products of each subtraction using two PCR reactions. The primary PCR was conducted in 25μl. It contained 1μl of diluted, subtracted cDNA, 1μl of PCR primer P1 (5μM), 1μl of PCR primer P2 (5μM), and 22μl of PCR master mixture prepared using the Advantage cDNA PCR Core Kit (Clontech). PCR was performed with the following parameters: 75°C for 7 min; 30 cycles at 91°C for 30 sec, 68°C for 30 sec, and 72°C for 2.5 min; and a final ex-

tension at 68°C for 7 min. The amplified products were diluted 10-fold in deionized water. One microliter of the product was then used as the template in a second PCR for 10 cycles under the same conditions used for the primary PCR, except PCR primers P1 and P2 were replaced with nested PCR primers PN1 and PN2, respectively. PCR was performed for 17 cycles of 91°C, 30 sec, 60°C, 30 sec, and 72°C, 2.5 min. The PCR products were analyzed by 2% agarose gel electrophoresis.

4. Differential screening (reverse Northern analysis)

The subtracted PCR products were inserted into the T/A cloning vector pCR2.1 (Invitrogen). Several hundred white colonies containing subtracted cDNA fragments grew in the X-gal/IPTG color assay. In all, 192 randomly picked clones (96 each from the forward- and reverse-subtracted libraries) were inoculated onto 96-well microtiter plates containing 100μl of Luria-Bertani (LB) medium and 50μg/ml ampicillin at 37°C, for 4 h with shaking at 225 rpm. One microliter of growing culture was transferred to a 0.2 ml PCR reaction tube containing the master mix with the secondary PCR primers and used as the PCR template. After PCR amplification, the PCR products were electrophoresed on a 1% agarose/ethidium bromide gel to verify that each recombinant contained the appropriate insert. Eighty-eight of each of the 96 PCR products from the forward- and reverse-subtracted clones were selected, and 8 PCR products each of β-actin and GAPDH were mixed with an equivalent volume of 0.6 N NaOH to denature them. Two microliters of denatured PCR products were transferred to nylon membranes. A set of two identical membranes with 96 dots was made using the forward- and reverse-subtracted libraries. Then, the two membranes were hybridized with a 32P-labeled forward/reverse-subtracted cDNA probe at 68°C. After hybridization, the membranes were washed with 2×SSC/0.5% SDS and 0.2×SSC/0.5% SDS solution and then exposed to X-ray film. The criteria for a positive blot result were as follows: 1) clones that hybridized to the forward-subtracted probe, but not to the reverse-subtracted probe in differential screening of the forward-subtracted library, and 2) clones that strongly hybridized to the reverse-subtracted probe, but not to the forward-subtracted probe in differential screening of the reverse-subtracted library. Positive clones identified in the differential screening were used for sequencing and Northern blotting.

5. DNA sequencing and gene identification

Plasmid DNA of the differentially expressed forward and reverse-subtracted clones in the PCR-select differential screening was extracted using a Wizard Plus Minipreps Kit (Promega) and then amplified by PCR using M13 reverse and forward primers. The amplified PCR fragments were electrophoresed in 1% agarose gels to purify them and then sequenced manually using the chain termination reaction or with an automatic DNA sequencer. Nucleic acid homology searches were performed using the BLAST program at the National Center for Biotechnology Information (National Institutes of Health, Bethesda).

6. Northern blot analysis

To confirm the differentially expressed genes seen in the dot blot differential screening, Northern blotting was performed. Probes were made from the PCR products that were differentially expressed in dot blot screening with a random labeling kit using ^{32}P. Fifteen micrograms each of total RNA from irradiated and non-irradiated HeLa cells were electrophoresed on 1.2% agarose formaldehyde gels and transferred to nylon membranes. The membranes were hybridized at 60°C for 18 h, and then washed twice at room temperature with 2×SSC and 0.1% SDS for 15 min and 0.1×SSC and 0.1% SDS at 60°C for 30 min. Autoradiographs were made by exposing the membrane to X-ray film at -70°C for 24 h to 7 days with an intensifying screen. Densitometric analyses were quantified using ImageQuant software, version 3.3 (Molecular Dynamics, Inc.). The densitometric results were standardized to that of β-actin expression in the same sample.

Results

A dot blot screening test was performed for the 88 randomly selected PCR products from the forward- and reverse-subtracted clones. The differentially expressed genes are shown in Figures 1 and 2. Duplicate blots were hybridized with forward- and reverse-subtracted probes. Fourteen clones had increased expression in the forward-subtracted library (irradiated HeLa cells) and 21 genes had increased expression in the reverse-subtracted library (non-irradiated HeLa cells). Some

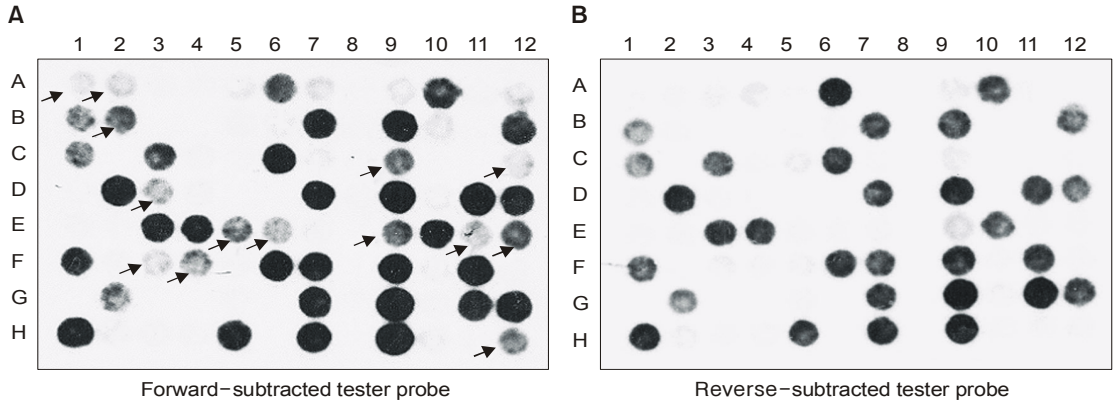

Fig. 1. Differential screening of the forward-subtracted cDNA library. Eighty-eight randomly selected colonies were amplified from the T/A cloning vector using adaptor-specific primers. After duplicating the membranes blotted with the forward-subtracted (irradiated HeLa cell) product, each was hybridized with (A) forward- and (B) reverse-subtracted probes. Lane 8 containsβ-actin and GAPDH as a negative control. The clones indicated with an arrow were selected.

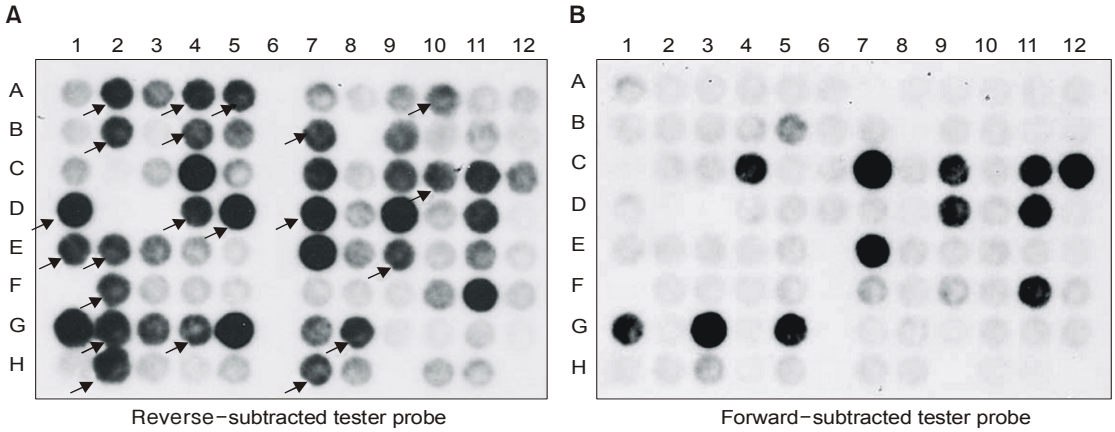

Fig. 2. Differential screening of the reverse-subtracted cDNA library. Eighty-eight randomly selected colonies were amplified from the T/A cloning vector using adaptor-specific primers. After duplicating the membranes blotted with the reverse-subtracted (non-irradiated HeLa cell) product, each was hybridized with (A) reverse- and (B) forward-subtracted probes. Lane 6 containsβ-actin and GAPDH as a negative control. The clones indicated with an arrow were selected.

Table 1. Differentially Expressed Genes in Forward-subtracted Clones

Clone	Size (bp)	Blast search with DNA sequences	GenBank No.
T 1-A	92,564	*Homo sapiens* telomerase catalytic subunit (TERT) and sodium channel-like protein genes.	AY007685
T 2-A	2,165	*Homo sapiens* cisplatin resistance related protein CRR9p (CRR9), mRNA	NM_030782
T 3-D	1,849	Human LIS mRNA (lissencephaly-1 protein)	L25107
T 3-F	994	*Homo sapiens*, ribosomal protein L12, clone MGC: 9760 IMAGE: 3855674, mRNA	BC008230
T 4-F	3,404	*Homo sapiens* heterogenous nuclear ribonocleoprotein D-like (HNRPDL), mRNA	XM_035993
T 5-E	2,801	*Homo sapiens*, clone IMAGE:3831740, mRNA	BC013869
T 9-C	1,719	*Homo sapiens* CREBBP/EP300 inhibitary protein 1 (CR11)	NM_014335
T 11-E	2,000	*Homo sapiens*, RAP 1B, member of RAS oncogene family	BC000176
T 12-E	2,638	*Homo sapiens* cDNA: FLJ22515 fis, clone HRC12122, highly similar to AF052101 Homo sapiens clone 23872 mRNA sequence	AK026168
T 12-H	803	*Homo sapiens* ribosomal protein S18/S6-like mRNA	AY090769

Table 2. Differentially Expressed Genes in Reverse-subtracted Clones

Clone	Size (bp)	Blast search with DNA sequences	GenBank No.
D 1-D	713	*Homo sapiens* similar to PEPSIN A PRECURSOR (LOC144064), mRNA	XM_084710
D 2-E	14,709	Human MEN1 region clone epsilon/beta mRNA, 3′ fragment	AF001893
D 2-G	3,273	*Homo sapiens* gamma-butulin complex component GCP5	AF272884
D 2-H	1,760	*Homo sapiens* HIF-1 responsive RTP801 (RTP801), mRNA	NM_019058
D 4-D	96,013	Human DNA sequence from clone RP-1-43017 on chromosome 1	AL353779
D 5-D	2,222	*Homo sapiens* keratin 6C (KRT6C)	NM_058242
D 7-H	1,358	*Homo sapiens* similar to Pepsin A precursor (LOC255721), mRNA	XM_170678
D 8-G	94,835	Human DNA sequence from clone 316D7 on chromosome 11p13. Contains parts of the gene for G2 protein, ESTs, STSs, and GSSs	AL049575
D 10-A	12,206	*Homo sapiens* multiple endocrine neoplasia I (MEN1), mRNA	XM_167804

genes overlapped in the subtracted clones. Three each from the forward- and reverse-subtracted libraries were not sequenced. Consequently, there were 10 unique differentially expressed genes in the forward-subtracted library and 9 in the reverse-subtracted library. The results of sequencing and a GenBank homology search in both directions of the subtracted libraries are described in Tables 1 and 2. The identified genes ranged in size from 803 to 92,564 bp in the forward-subtracted library and from 713 to 94,835 bp in the reverse-subtracted library. The differentially expressed genes in the forward-subtracted cDNA library were as follows: two with known sequences, but unknown functions, two ribosomal proteins, a gene associated with increased telomerase activity, a ribonucleoprotein, a cisplatin resistance-related protein, a gene associated with human lissencephaly, and an anti-mitogenic GTP binding protein. The differentially expressed genes in the reverse-subtracted cDNA library were as follows: two with known sequences, but unknown functions, two multiple endocrine neoplasia I genes, a gamma-tubulin complex, a hypoxia-inducible factor (HIF-1), and a keratin 6 protein.

To further confirm the genes showing differential expression on differential screening, 13 genes from the forward-subtracted library and 21 from the reverse-subtracted library were subjected to Northern blot analysis. Northern blotting confirmed increased mRNA expression in irradiated HeLa cells for three genes from the forward-subtracted clones: a human telomerase catalytic subunit and sodium channel-like protein gene (T 1- A), a human ESTs (T 5-E), and a human ribosomal protein S18/S6-like mRNA (T 12-H)(Fig. 3). No genes of the reverse-subtracted clones showed differential expression.

Northern blot analysis was used to evaluate the expression pattern of the T1-A and T5-E clones with irradiation dose. HeLa cells were subjected to graded radiation doses of 0.1, 0.5, 1, and 2 Gy. The T 5-E and T 1-A mRNA of irradiated HeLa cells showed a dose-dependent increase in mRNA expression, with markedly increased expression at a dose of 1 Gy (Fig. 4).

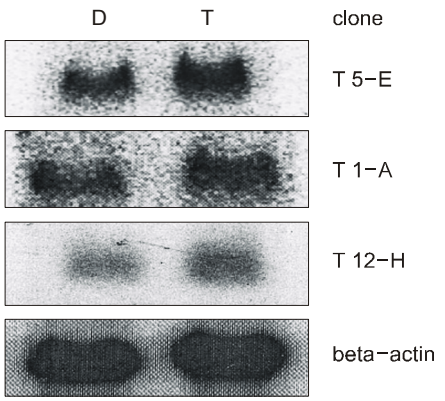

Fig. 3. Northern blot analysis of selected differentially expressed mRNA. Thirteen forward-subtracted clones with a differential signal detected in the screening were used for each cDNA probe for Northern blotting of mRNA isolated from irradiated (single dose of 10 Gy) and non-irradiated HeLa cells. Each lane contained 15μg/ml total RNA from irradiated or non-irradiated HeLa cells. Of the thirteen clones, four did not show a band and six did not differ between the tester and driver clones. Three clones showed differential expression in the irradiated and non-irradiated HeLa cells: T 5-E, T 1-A, and T12-H. *D*, driver (non-irradiated HeLa cells); *T*, tester (irradiated HeLa cells).β-actin expression was used as an internal control.

Discussion

There is no precise sensor for detecting the effects of ion-

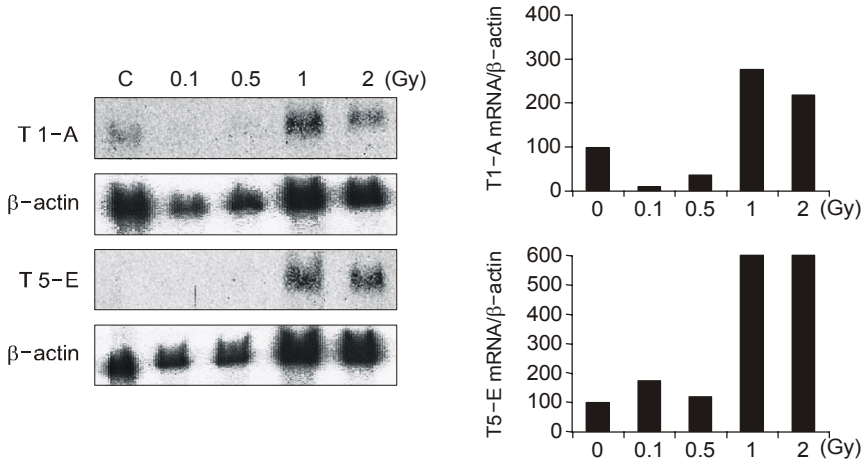

Fig. 4. Northern blot analysis of clones T1-A and T5-E according to the radiation dose. cDNA was used to probe Northern blots of mRNA isolated from irradiated HeLa cells. Clones T1- A and T5-E showed differential expression with increasing radiation dose. The radiation dose delivered to the HeLa cells was 0.1, 0.5, 1, and 2 Gy, respectively.β-actin expression was used as an internal control. The lower panel shows the densitometric analysis of the ratios of T1-A and T5-E mRNA toβ-actin with radiation dose.

izing radiation. However, several ways of recognizing radiation damage have been suggested, including 1) a nuclear-derived signal from damaged DNA, 2) a cytoplasmic factor, such as NF (B, or 3) a cellular membrane that might undergo lipid peroxidation or through which a transient ionic flux might be induced by radiation.[12] The signals caused by these mechanisms are mediated via signal transduction pathways, such as the protein kinase pathway. As a result, diverse early and late response radiation-induced genes are expressed.

One of the purposes of studying the expression of radiation-induced genes is to understand the cytotoxic effect of radiation on tumor cells by determining the molecular biological mechanisms involved. Furthermore, discovering a correlation between the expression patterns of radiation-induced genes and clinical outcome in cancer patients could be a useful method for predicting the prognosis of patients treated with radiation. Radiation-induced genes have various functions in tumor and normal cells. Early response genes to irradiation, such as c-*jun*, c-*fos*, and EGR-1, regulate transcriptional activity in cells.[13] Some cytokines and growth factors are involved in the pathogenesis of tissue inflammation and fibrotic changes in tissue following irradiation.[8,14]

Several methods can be used to isolate differentially expressed genes, including Northern blotting,[15] differential display (DD),[16,17] DNA microarray,[18] suppression subtractive hybridization (SSH),[10,11] oligonucleotide chip,[19] and serial analysis of gene expression.[20] In this study, we studied gene expression following irradiation using the SSH technique. One of the advantages of SSH is its powerful capacity to identify tissue-specific differentially expressed genes that are expressed in very low transcript numbers.[11] One potential disadvantage of SSH is that it requires a few micrograms of poly $(A)^+$ RNA from two cell populations. In some situations, such as getting tissue from patients undergoing radiation treatment, it may be difficult to obtain an adequate quantity of RNA because tumor size regresses with accumulation of the radiation dose. To resolve this problem, we used HeLa cells as a model of uterine cervix carcinoma in this study.

Of the 10 genes in the forward-subtracted cDNA library that showed differential expression on dot blot screening, only the human telomerase catalytic subunit (hTERT) and sodium channel-like protein gene, human ESTs, and human ribosomal protein S18/S6-like mRNA were confirmed by Northern blotting. No genes from the reverse-subtracted cDNA library were confirmed by Northern blotting. Therefore, we think that a complementary method, such as DD or DNA microarray, would help to clarify the cause of the difference between the screening test and the Northern blot result. The hTERT gene activates telomerase with telomere elongation, and there is a close relationship between hTERT expression and telomerase activity.[21,22] Telomerase activity is increased in cancer cells and immortal cell lines, but not in nonmalignant or normal cells. Consequently, many studies have examined the use of telomerase activity in the diagnosis of cancer and predicting treatment prognosis.[23~26] Sawant et al. found that telomerase activity was down-regulated in plateau phase HeLa cells after radiation exposure in a dose-dependent manner.[26] In contrast, growing HeLa cells irradiated with 10 Gy showed an increased level of telomerase activity, but there was no increase in the level of hTERT mRNA. Compared with our study,

there were some differences in the selection of HeLa cells (stationary phase vs. growing cells), and we did not perform an additional experiment to examine telomerase activity status. Nevertheless, our study indicated that hTERT was up-regulated in stationary phase HeLa cells in a dose-dependent manner. An ESTs gene (*Homo sapiens*, clone IMAGE: 3831740, mRNA) was also expressed in a dose-dependent manner, but its function is not yet known.

In the forward-subtracted cDNA library, cisplatin resistance related protein CRR9 is related to cisplatin resistance in tumor cells. Recently, Yamamoto et al. reported that CRR9 was increased in a cisplatin-resistant ovarian cancer cell line and was not associated with CDDP-resistance, but with CDDP-induced apoptosis.[27] It is known that low-dose fractionated irradiation (30 Gy/10 fractions) also induces moderate cisplatin resistance in HeLa cells.[28] RAP 1B is a member of the RAS oncogene family and is an anti-mitogenic GTP-binding protein. In a recent study, however, it was reported to be a conditional oncoprotein that also has mitogenicity.[29]

In the reverse-subtracted cDNA library, a hypoxia-inducible factor-1 (HIF-1) responsive gene, RTP801, was identified. This gene is strongly up-regulated by hypoxia.[30] In our study, the irradiation of HeLa cells resulted in decreased RTP801 gene expression. This suggests that the irradiation of cancer cells may improve oxygenation.

In summary, we identified 10 (2 with unknown function and 8 with known function) differentially expressed radiation-induced genes in irradiated HeLa cells and 9 (2 unknown function and 7 known function) genes in non-irradiated HeLa cells using SSH. These findings may provide important information to help understand the effects of radiation on tumor cells in uterine cervical carcinoma. We were able to identify low-abundance differentially expressed radiation-induced genes in HeLa cells with SSH, but further characterization of theses genes are necessary to clarify the biological functions of them.

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국문초록

자궁경부암세포에서 방사선조사시 차등 발현되는 유전자 동정

충남대학교 의과대학 방사선종양학교실*, 피부과교실†, 자연과학대학 생물학과‡, 암공동연구소§

김준상*§ · 이영숙§ · 이증훈† · 이웅희‡ · 서은영† · 조문준*§

목적: 자경경부암세포에서 polymeric chain reaction (PCR)원리를 이용한 suppression subtractive hybridization (SSH) 방법으로 방사선조사 시 차등 발현되는 유전자를 동정하고자 하였다.

대상 및 방법: 자궁경부암세포주인 HeLa 세포주에 방사선조사 전과 후 총 RNA와 poly $(A)^+$ mRNA를 분리하였다. SSH방법으로 forward 및 reverse-subtracted cDNA libraries를 만들었다. 차등 발현된 유전자를 screening하기 위해 reverse Northern blotting (dot blot analysis)을 이용하여 각각의 library에서 88개의 클론을 선택하였고 Nothern blotting으로 확인 후 sequencing하였다.

결과: screening상 176개 클론 중 forward-subtracted library에서 10개의 유전자가 reverse-subtracted library에서 9개의 유전자가 동정되었다. forward-subtracted library로부터 3개의 유전자가 Northern blotting에 의하여 확인되었고 이중 telomerase catalytic subunit and sodium channel-like protein 유전자와 1개의 ESTs (expressed sequence tags) 유전자가 방사선선량에 따라 증가하였다.

결론: 본 연구를 통해 자궁경부암세포주에서 방사선에 의해 유도되는 유전자를 SSH 방법을 통해 동정할 수 있었다. 그러나 이러한 유전자가 어떤 생물학적인 기능을 갖고 있는지에 대한 계속적인 연구가 필요하다.

핵심용어: 자궁경부암, Suppression subtractive hybridization, HeLa 세포, 방사선유도유전자