Identification of a radiosensitivity gene signature in gastric cancer cells using microarray analysis

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Directed by Professor Sun Young Rha

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Han Sang Kim

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

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ABSTRACT

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Background: Prediction of response prior to radiotherapy is future direction of radiotherapy and identification of druggable targets in radiotherapy could overcome resistance. In order to identify a radiosensitivity gene signature and elucidate relevant signaling pathways, microarrays using gastric cancer cells were analyzed before radiotherapy.
Methods: Oligonucleotide microarray containing 22,740 probes was performed using twelve gastric cancer cells before radiation. Clonogenic assays with 2Gy of radiation were performed and survival fraction at 2 Gy (SF2) was measured as surrogate marker for radiosensitivity. Differentially expressed genes were identified between radiosensitive and radioresistant cells and gene set analysis was performed. Pathway analysis using Ingenuity pathway analysis (IPA) was conducted. Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) was performed for validation.
Results: In individual gene analysis, 68 genes were identified as a radiosensitivity gene signature. Identified genes were interact with VEGF, AKT, TGF-β, NFκB, ERK, PI3K, HIF1A, MDM2, TGFB1 and TP53 in IPA. Functions associated with genetic networks were cellular growth and proliferation, cellular movement, and cell cycle. Gene set analysis using entire genes enriched several pathways including Akt signaling. qRT-PCR results were well correlated with microarray experiments (the

Pearson correlation coefficient, 0.91-0.99).

Conclusion: We first identified 68-radiosensitivity gene signature in gastric cancer cells. Akt signaling pathway could be druggable target for radiosensitization in gastric cancer. We suggest that this analysis could elucidate targets for radiosensitivity biomarker discovery and the identified genes and signaling pathways could be served as potential targets in radiotherapy.

Key words : radiosensitivity; gastric cancer; gene expression profiling; clonogenic assay; akt

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I. INTRODUCTION

Prediction of response prior to radiotherapy is one of future direction in radiation oncology. Despite several clinical factors such as stage, tumor size, histology, and the status of resection margin helps deciding treatment modality and schedule, clinical parameters alone are not accurate and sufficient to explain heterogeneity among the patients.[1,2] Moreover, biological factors that determine response seemed exist, so called intrinsic radiosensitivity.[3] Beyond technical development regarding best fractionation and dose schedule, identification and development of biomarkers for intrinsic radiosensitivity could explain radiobiology and reliable prediction.

Molecular biomarkers include genetic mutation, gene expression, and genomic loss/amplification and have been suggested in several cancer types including colorectal,[4-6] lung,[7] and head and neck cancers.[8,9] The value of biomarkers includes decision of treatment modality, prognosis, and prediction of drug response or toxicity as well as understanding intrinsic radiosentivity. Of note, recently developed molecular-targeted agents blocking specific signaling pathway could be effectively treated according to molecular profile.[10-13]

Gene expression profiles using microarray technology in radiosensitivity has been conducted in various types of cancers including colorectal, head and neck, cervical, and breast cancer.[5,6,14-17] As measuring the expression level of

thousands of genes simultaneously enables identification of genes, cellular function, or relevant signaling pathways, this approaches are promising in identifying new possible targets in addition to suggested predictive markers such as *p53*, *cyclin D1*, bcl-2, Ki-67, and vascular endothelial growth factor.[18]

Gastric cancer (GC) is the second leading cause of cancer-related death worldwide (738,000 deaths, 9.7% of the total) in patients with cancer.[19] As loco-regional recurrence is significant with prognosis in GC patients, the role of radiotherapy in GC has been increased and perioperative radiotherapy has been evaluated.[20-22] Preoperative radiotherapy improved resection rate and survival[21] and postoperative radiotherapy showed better loco-regional recurrence.[20] Accordingly, there is an increasing attraction for improving loco-regional control and survival.

In this article, we measured radiosensitivity index (survival fraction at 2 Gy of radiation, SF2), analyzed mRNA expression profiling of gastric cancer cells before radiotherapy, and identified differentially expressed genes, biological functions, and relevant signaling pathways by comparing radiosensitive and radioresistant cells.

II. MATERIALS AND METHODS

Cell lines and culture

Twelve gasric cell lines (AGS, MKN-1, MKN-74, SNU-216, SNU-484, SNU-638, YCC-1, YCC-16, YCC-2, YCC-3, YCC-6, YCC-7) were used. SNU-series were obtained from Korean cell line bank, and YCC-series were established from Korean gastric cancer patients at the Cancer Metastasis Research Center (CMRC, Yonsei University College of Medicine, Seoul, Korea). Cells were cultured under conditions provided by the manufacturer, and were incubated at 37 ℃ in a 5% CO_2 humidified atmosphere and the media replaced every 3 days.

Radiation survival clonogenic assays (survival fraction at 2 Gy, SF2)

To evaluate radiation sensitivity in gastric cancer cells, cells were plated in triplicate so that 100 to 200 colonies would form per plate and incubated overnight at 37℃ to allow adherence. Cells were then radiated with X-rays of 2Gy. After fixation, colonies over 50 cells were calculated. Experiments replicate three times independently and average value was used. SF2 was determined by the formula (SF2 = number of colonies / total numbers of cells plated × plating efficiency).

RNA preparation and oligonucleotide microarrays

Total RNA was extracted from each cell line using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The Yonsei reference RNA was prepared [23]. The quantity and quality of RNA were confirmed by a ND-1000 spectrophotometer (NanoDrop Technologies, USA) and gel electrophoresis. Oligonucleotide microarray analysis was performed using a human oligo chip (CMRC-GT, Seoul, Korea) containing 22,740 oligonucleotide probes of 70 bases with a reference design. The test samples (RNA from each gastric cancer cell) were labeled with Cy5 and individually co-hybridized with the Cy3-labeled reference RNA (CMRC, Seoul, Korea).

Data and statistical analysis

Microarray data extraction and analysis were done using BRB-ArrayTools (http://linus.nci.nih.gov/BRB-ArrayTools.html) for class comparison and gene set analysis. Differentially expressed genes (DEGs) between radiosensitive and radioresistant cells were identified using a random-variance t test ($P<0.05$). P value was adjusted for multiple hypothesis testing using q-value suggested by Storey[24]. Gene set analysis was performed using Biocarta pathways which are obtained from the Cancer Genome Anatomy Project (http://cgap.nci.nih.gov/Pathways). Genetic network was generated through the

use of Ingenuity Pathways Analysis (IPA, Ingenuity Systems, www.ingenuity.com). DEGs were overlaid onto a global molecular network developed from information contained in the Ingenuity Knowledge Base. Networks of network eligible molecules were then algorithmically generated based on their connectivity.

Quantitative RT-PCR

DIRAS3, *CDKN2B*, *POF1B*, *ALDH1A1*, and *ANTXR2* were selected for validation of the microarray data. Quantitative RT-PCR (qRT-PCR) was performed on 12 gastric cancer cells. In brief, 4μg of total RNA from each sample was reverse-transcribed using SuperScript II Reverse Transcriptase (Invitro-gen, Carlsbad, CA, USA). Two hundred nanograms of synthesized cDNA were PCR amplified using QuantiTect SYBR Green PCR (QIAGEN, Valencia, CA, USA). Each reaction was run on a Stratagene MX3005P (Stratagene, La Jolla, CA, USA). Expression values for each gene were determined using a standard curve constructed from Human Genomic DNA (Promega, Madison, WI, USA). The house-keeping gene *HPRT* was selected for normalization and the standard curve. Non-template-control wells without cDNA were included as negative controls. The primer sets for PCR amplification were designed.

Table 1. Primer sequences of five genes of 68-radiosensitive gene signature for quantitative real-time PCR experiments.

Accession Number	Gene symbol	Primer Sequence	Product Size (bp)
NM_004675	DIRAS3	Forward:CCAACACCACTGAGAAGCTG Reverse:CACGTTTTCTACACGCTACAGG	97
NM_078487	CDKN2B	Forward:GGTGCACTGCTTTGGGATT Reverse:CCCACCTCTTGGAGTTCAAT	115
NM_058172	ANTXR2	Forward:AGCGATTGGAGCATCCTG Reverse:GTGCCACAAACCTGGACAC	100
NM_024921	POF1B	Forward:CGGCTAAAATGTTAAGCTCCA Reverse:TCCTCCGTTGTTCCTACACC	138
NM_000689	ALDH1A1	Forward:GCTTCTTTCCCTTAGTGACTCTTG Reverse:GCAGACATGACATCCTAGGAAAC	127

III. RESULTS

Survival fraction at 2Gy of radiation in GC cells

Study scheme was represented in Figure 1. To determine radiosensitivity of gastric cells, twelve GC cells were irradiated at 2 Gy and clonogenic assays were performed (Figure 2). SNU-638 and MKN-1 cells were highly radiosensitive than others (SF2; 0.127 and 0.143, respectively). YCC-2, YCC-16, and YCC-7 cells showed SF2 of more than 0.6 (SF2; 0.609, 0.620, 0.667, respectively). Doubling time, PIK3CA mutation status, and KRAS mutation status were not associated with SF2 (Table 2).

Figure 1. Study overview

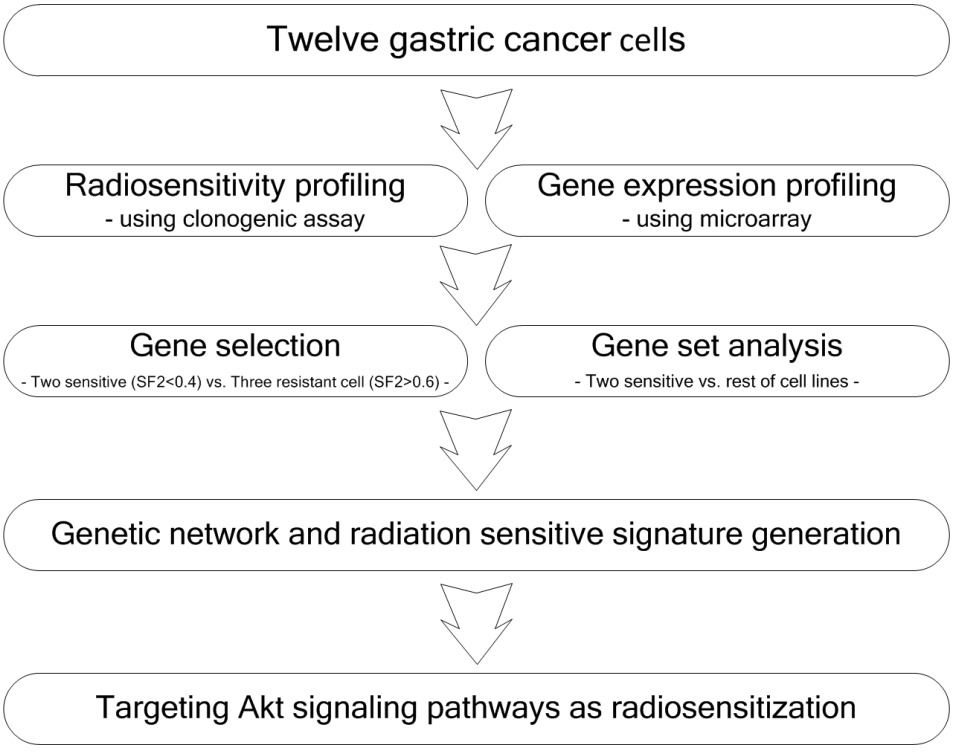


Figure 2. Survival fraction at 2 Gy (SF2) in twelve gastric cancer cells

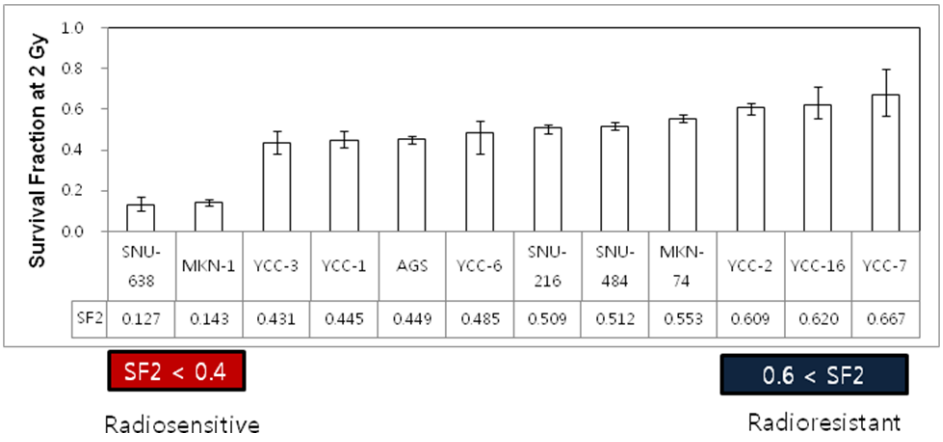


Table 2. Characteristics of 12 gastric cancer cells

Cell line	SF2	Doubling time (h)	PIK3CA mutation	KRAS mutation
SNU-638	0.127	25	-	-
MKN-1	0.143	31	+	-
YCC-3	0.431	34	-	-
YCC-1	0.445	40	-	-
AGS	0.449	20	-	+
YCC-6	0.485	48	-	-
SNU-216	0.509	36	-	-
SNU-484	0.512	34	-	-
MKN-74	0.553	32	-	-
YCC-2	0.609	43	-	+
YCC-16	0.620	22	+	-
YCC-7	0.667	35	-	-

Abbreviation: SF2, survival fraction at 2 Gy of radiation.

Identification of a gene signature and gene set analysis between radiosensitive and radioresistant GC cells

To identify individual genes and function relevant to radiosensitivity, gene expression before radiation was measured. Class comparison between radiosensitive cells (SF2 $\leq$ 0.4) and radiosensitive cells (SF2 $\geq$ 0.6) identified 613 genes which showed different expression level of more than 2-fold (Figure 3A). Of these genes, 68 genes showed expression changes more than 6-fold (Table 3). In clustering analysis, radiosensitive cells (SNU-638 and MKN-1) were discriminated from other cells, range of SF2 from 0.431 to 0.667 (Figure 3B).

Functional annotation and pathway analysis of identified 68-gene signatures was performed using Ingenuity pathway analysis (IPA). Figure 4 showed top four genetic networks enriched in pathway analysis. Each genetic network showed interaction via major signaling pathway molecules including *VEGF, AKT, TGF-β, NFκB*, and *ERK* (Figure 4A, IPA score 56), *PI3K*, *HIF1A*, and *MDM2* (Figure 4B, IPA score 28), *TGFB1* (Figure 4C, IPA score 26), and *TP53* (Figure 4D, IPA score 23). Functions associated with genetic networks were cellular growth and proliferation, cellular movement, and cell cycle.

Concurrently with individual gene identification, gene set analysis was performed with entire genes and summarized in Table 4. Several signaling pathways were enriched including p38 MAP kinase signaling, Akt signaling, tumor suppressor ARF (alternative reading frame) signaling pathways. For the integration of individual gene identification and gene set analysis, commonly enriched Akt signaling pathway was selected as the target pathway regarding radiosensitivity. Nine genes of 68-genes were related to Akt signaling pathway and summarized in Table 5.

Figure 3. Comparison of gene expression profiling (A) between radiosensitive (SNU-638 and MKN-1) and radioresistant cells (YCC-7, YCC-2, and YCC-16) and (B) in 12 gastric cells (the genes with a more than 2-fold increase shown in red while those with a more than 2-fold decrease shown in green)

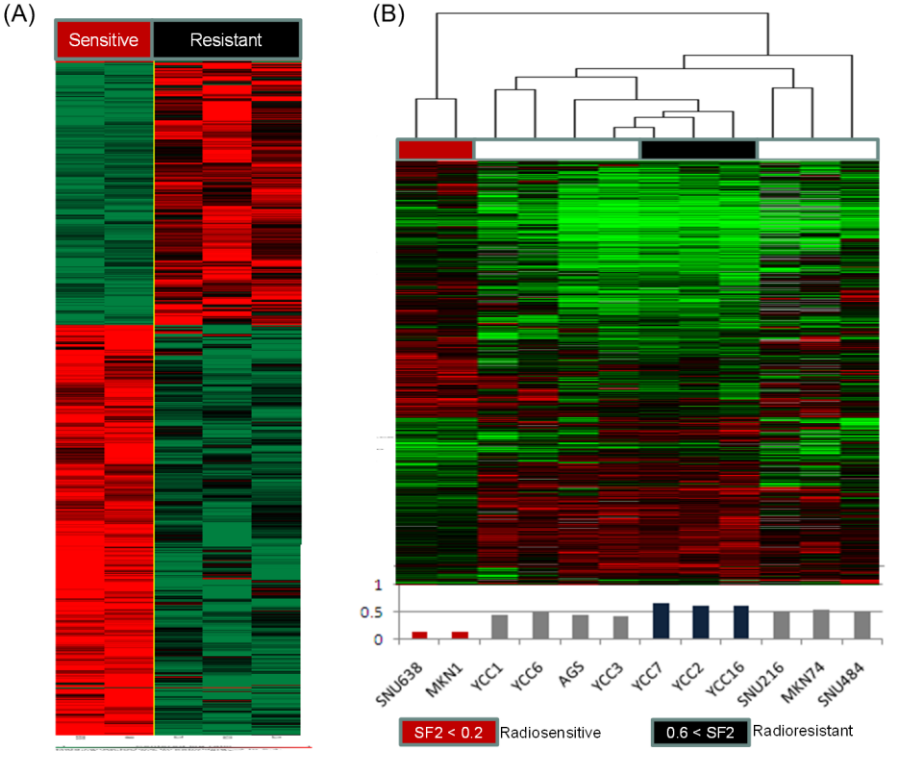


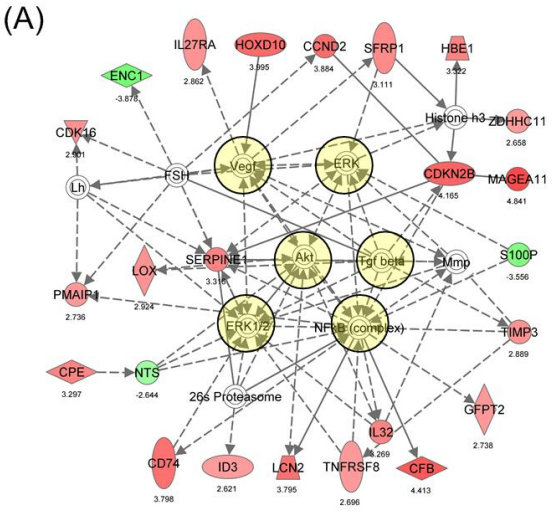
Table 3. List of discriminating genes between radiosensitive and radioresistant cells showing expression changes more than 6 folds

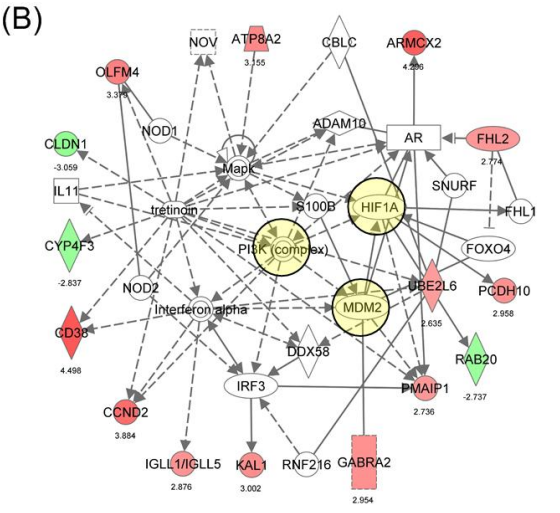
Accession	Gene symbol	Gene name	Fold-change (Radiosensitive / Radioresistant)	Adjusted q-value[24]
NM_004675	DIRAS3	DIRAS family, GTP-binding RAS-like 3	45.15	0.003
NM_005366	MAGEA11	Melanoma antigen family A, 11	28.67	0.009
NM_001775	CD38	CD38 molecule	22.60	0.014
NM_024728	C7orf10	Chromosome 7 open reading frame 10	21.46	0.005
NM_001710	CFB	Complement factor B	21.31	0.009
NM_014782	ARMCX2	Armadillo repeat containing, X-linked 2	19.65	0.009
NM_078487	CDKN2B	Cyclin-dependent kinase inhibitor 2B (p15, inhibits CDK4)	17.94	0.008
XM_051522		ESTs	16.09	0.021
NM_021192	HOXD11	Homeobox D11	16.02	0.006
NM_002148	HOXD10	Homeobox D10	15.94	0.010
NM_004165	RRAD	Ras-related associated with diabetes	15.02	0.019
NM_001759	CCND2	Cyclin D2	14.76	0.006
NM_004355	CD74	CD74 molecule, major histocompatibility complex, class II invariant chain	13.91	0.013
NM_005564	LCN2	Lipocalin 2	13.88	0.019
NM_021992	TMSB15A	Thymosin beta 15a	13.77	0.002
NM_015967	PTPN22	Protein tyrosine phosphatase, non-receptor type 22 (lymphoid)	12.64	0.006
NM_058172	ANTXR2	Anthrax toxin receptor 2	12.28	0.015
NM_005723	TSPAN5	Tetraspanin 5	11.03	0.008
NM_002728	PRG2	Proteoglycan 2, bone marrow (natural killer cell activator, eosinophil granule major basic protein)	11.00	0.001
XM_072568		ESTs	10.63	0.001
NM_004784	NDST3	N-deacetylase/N-sulfotransferase (heparan glucosaminyl) 3	10.45	0.001
NM_006418	OLFM4	Olfactomedin 4	10.40	0.003
NM_016606	REEP2	Receptor accessory protein 2	10.11	0.006
NM_005330	HBE1	Hemoglobin, epsilon 1	10.00	0.004
XM_059689		ESTs	10.00	0.003

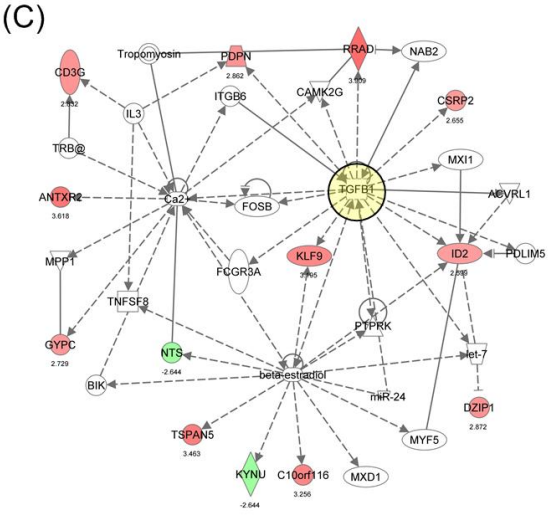
NM_000602	SERPINE1	Serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1	9.96	0.001
NM_001873	CPE	Carboxypeptidase E	9.83	0.013
NM_004221	IL32	Interleukin 32	9.64	0.002
NM_006829	C10orf116	Chromosome 10 open reading frame 116	9.55	0.012
NM_032413	C15orf48	Chromosome 15 open reading frame 48	9.25	0.006
NM_001206	KLF9	Kruppel-like factor 9	9.16	0.013
XM_117239		ESTs	9.08	0.009
NM_016529	ATP8A2	ATPase, aminophospholipid transporter, class I, type 8A, member 2	8.91	0.009
NM_003012	SFRP1	Secreted frizzled-related protein 1	8.64	0.001
NM_006096	NDRG1	N-myc downstream regulated 1	8.24	0.004
NM_000216	KAL1	Kallmann syndrome 1 sequence	8.01	0.007
NM_032961	PCDH10	Protocadherin 10	7.77	0.006
NM_000807	GABRA2	Gamma-aminobutyric acid (GABA) A receptor, alpha 2	7.75	0.021
NM_004445	EPHB6	EPH receptor B6	7.65	0.013
NM_002317	LOX	Lysyl oxidase	7.59	0.004
NM_033018	CDK16	Cyclin-dependent kinase 16	7.47	0.013
NM_000362	TIMP3	TIMP metallopeptidase inhibitor 3	7.41	0.007
NM_020070	IGLL1	Immunoglobulin lambda-like polypeptide 1	7.34	0.001
NM_014934	DZIP1	DAZ interacting protein 1	7.32	0.006
NM_006474	PDPN	Podoplanin	7.27	0.008
NM_004843	IL27RA	Interleukin 27 receptor, alpha	7.27	0.005
NM_000073	CD3G	CD3g molecule, gamma (CD3-TCR complex)	7.12	0.001
NM_001450	FHL2	Four and a half LIM domains 2	6.84	0.016
NM_005110	GFPT2	Glutamine-fructose-6-phosphate transaminase 2	6.67	0.009
NM_021127	PMAIP1	Phorbol-12-myristate-13-acetate-induced protein 1	6.66	0.006
NM_002101	GYPC	Glycophorin C (Gerbich blood group)	6.63	0.002
XM_173012		ESTs	6.60	0.003
NM_005024	SERPINB10	Serpin peptidase inhibitor, clade B (ovalbumin), member 10	6.53	0.003

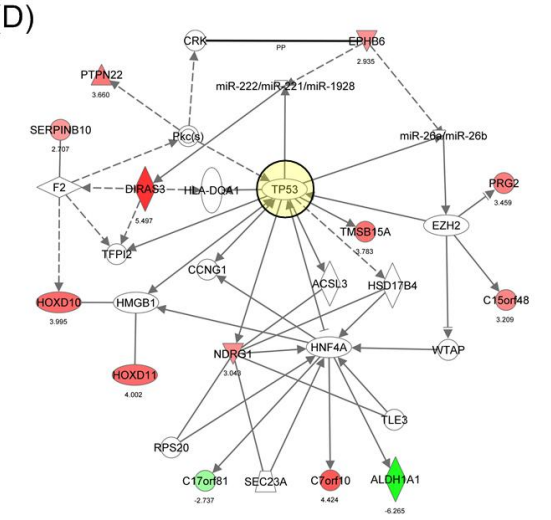
NM_001243	TNFRSF8	Tumor necrosis factor receptor superfamily, member 8	6.48	0.006
NM_017826	SOHLH2	Spermatogenesis and oogenesis specific basic helix-loop-helix 2	6.37	0.016
NM_024786	ZDHHC11	Zinc finger, DHHC-type containing 11	6.31	0.014
NM_001321	CSRP2	Cysteine and glycine-rich protein 2	6.30	0.003
NM_004223	UBE2L6	Ubiquitin-conjugating enzyme E2L 6	6.21	0.006
NM_002167	ID3	Inhibitor of DNA binding 3, dominant negative helix-loop-helix protein	6.15	0.016
NM_138461	TM4SF19	Transmembrane 4 L six family member 19	6.11	0.014
XM_067948		ESTs	6.08	0.004
NM_002166	ID2	Inhibitor of DNA binding 2, dominant negative helix-loop-helix protein	6.06	0.006
XM_166314		ESTs	6.05	0.014
NM_153425		ESTs	0.17	0.004
NM_006183	NTS	Neurotensin	0.16	0.001
NM_003937	KYNU	Kynureninase	0.16	0.006
NM_017817	RAB20	RAB20, member RAS oncogene family	0.15	0.006
NM_015362	C17orf81	Chromosome 17 open reading frame 81	0.15	0.009
NM_000896	CYP4F3	Cytochrome P450, family 4, subfamily F, polypeptide 3	0.14	0.002
NM_021101	CLDN1	Claudin 1	0.12	0.012
NM_014399	TSPAN13	Tetraspanin 13	0.10	0.010
NM_005980	S100P	S100 calcium binding protein P	0.09	0.003
NM_016613	FAM198B	Family with sequence similarity 198, member B	0.07	0.010
NM_003633	ENC1	Ectodermal-neural cortex 1 (with BTB-like domain)	0.07	0.010
NM_024921	POF1B	Premature ovarian failure, 1B	0.05	0.001
NM_000689	ALDH1A1	Aldehyde dehydrogenase 1 family, member A1	0.01	0.001

Figure 4. Pathway analysis using 68-identified genes associated radiosensitivity from the Ingenuity Pathway Analysis (IPA) Interactive network through (A) VEGF, AKT, TGF-β, NFκB, and ERK (B) PI3K, HIF1A, and MDM2 (C) TGFB1 (D) TP53 (E), and (F) Supporting information including genes, IPA score, and relevant functions for each network (A) through (D) (IPA network for recursive partitioning prioritized genes. Genes with red node are up-regulated genes in 68-radiosensitivity gene signature in radiosensitive cells while genes with green node are down-regulated in our analysis, others are generated through the network analysis from the Ingenuity Pathways Knowledge Base (http://www.ingenuity.com). Edges are displayed with labels that describe the nature of the relationship between the nodes. All edges are supported by at least one reference from the literature, or from canonical information stored in the Ingenuity Pathways Knowledge Base. Edges are displayed with labels that describe the nature of the relationship between the nodes. The lines between genes represent known interactions, with solid lines representing direct interactions and dashed lines representing indirect interactions. Nodes are displayed using various shapes that represent the functional class of the gene product. Nodes are displayed using various shapes that represent the functional class of the gene product)









(E)

ID	Molecules in Network	IPA score	No. of genes from 68-gene signature	Top Functions
A	26s Proteasome, Akt, ↑**CCND2**, ↑**CD74**, ↑**CDK16**, ↑**CDKN2B**, ↑**CFB**, ↑**CPE**, ↓**ENC1**, ERK, ERK1/2, FSH, ↑**GFPT2**, ↑**HBE1**, Histone h3, ↑**HOXD10**, ↑**ID3**, ↑**IL32**, ↑**IL27RA**, ↑**LCN2**, Lh, ↑**LOX**, ↑**MAGEA11**, Mmp, NFkB, ↓**NTS**, ↑**PMAIP1**, ↓**S100P**, ↑**SERPINE1**, ↑**SFRP1**, Tgf beta, ↑**TIMP3**, ↑**TNFRSF8**, Vegf, ↑	56	24	Cancer, Cellular Movement, Cellular Growth and Proliferation
B	ADAM10, AR, ↑**ARMCX2**, ↑**ATP8A2**, CBLC, ↑**CCND2**, ↑**CD38**, ↓**CLDN1**, ↓**CYP4F3**, DDX58, FHL1, ↑**FHL2**, FOXO4, ↑**GABRA2**, HIF1A, ↑**IGLL1/IGLL5**, IL11, Interferon alpha, IRF3, ↑**KAL1**, Mapk, MDM2, NOD1, NOD2, NOV, ↑**OLFM4**, ↑**PCDH10**, PI3K , ↑**PMAIP1**, ↓**RAB20**, RNF216, S100B, SNURF, tretinoin, ↑	28	15	Cell-To-Cell Signaling and Interaction, Cellular Movement, Gene Expression
C	ACVRL1, ↑**ANTXR2**, beta-estradiol, BIK, ↑**C10orf116**, CAMK2G, ↑**CD3G**, ↑**CSRP2**, ↑**DZIP1**, FCGR3A, FOSB, ↑**GYPC**, ↑**ID2**, IL3, ITGB6, ↑**KLF9**, ↓**KYNU**, miR-24, MPP1, MXD1, MXI1, MYF5, NAB2, ↓**NTS**, PDLIM5, ↑**PDPN**, PTPRK, ↑**RRAD**, TGFB1, TNFSF8, ↑**TSPAN5**	26	13	Cell Cycle, Cellular Function and Maintenance, Cell-To-Cell Signaling and Interaction
D	ACSL3, ↓**ALDH1A1**, ↑**C15orf48**, ↓**C17orf81**, ↑**C7orf10**, CCNG1, CRK, ↑**DIRAS3**, ↑**EPHB6**, EZH2, F2, HLA-DQA1, HMGB1, HNF4A, ↑**HOXD10**, ↑**HOXD11**, HSD17B4, miR-222/miR-221/miR-1928, miR-26a/miR-26b, ↑**NDRG1**, Pkc, ↑**PRG2**, ↑**PTPN22**, RPS20, SEC23A, ↑**SERPINB10**, TFPI2, TLE3, ↑**TMSB15A**,	23	13	Cellular Movement, Cancer, Cellular Growth and Proliferation

(F)

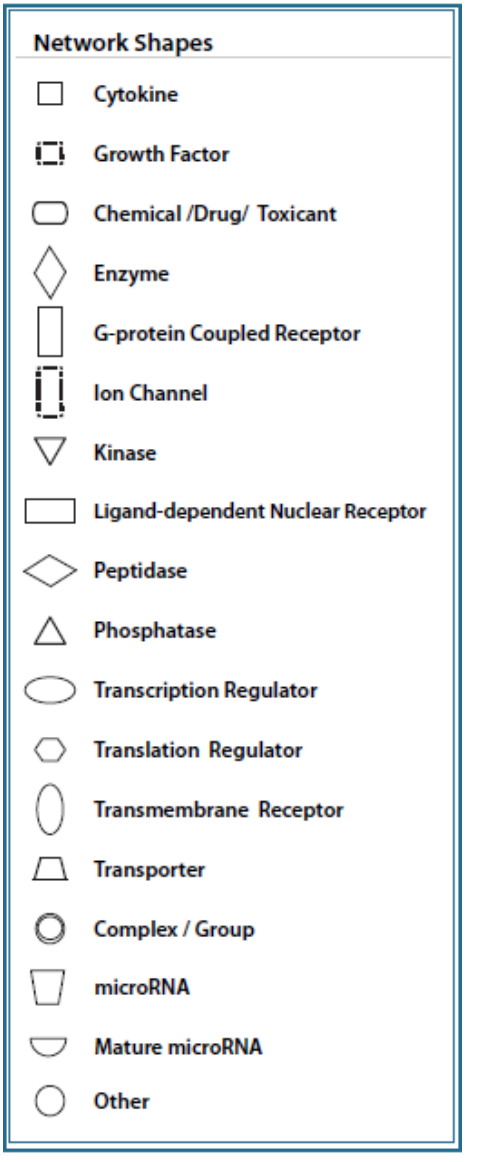

Network Shapes
Cytokine
Growth Factor
Chemical /Drug/ Toxicant
Enzyme
G-protein Coupled Receptor
Ion Channel
Kinase
Ligand-dependent Nuclear Receptor
Peptidase
Phosphatase
Transcription Regulator
Translation Regulator
Transmembrane Receptor
Transporter
Complex / Group
microRNA
Mature microRNA
Other

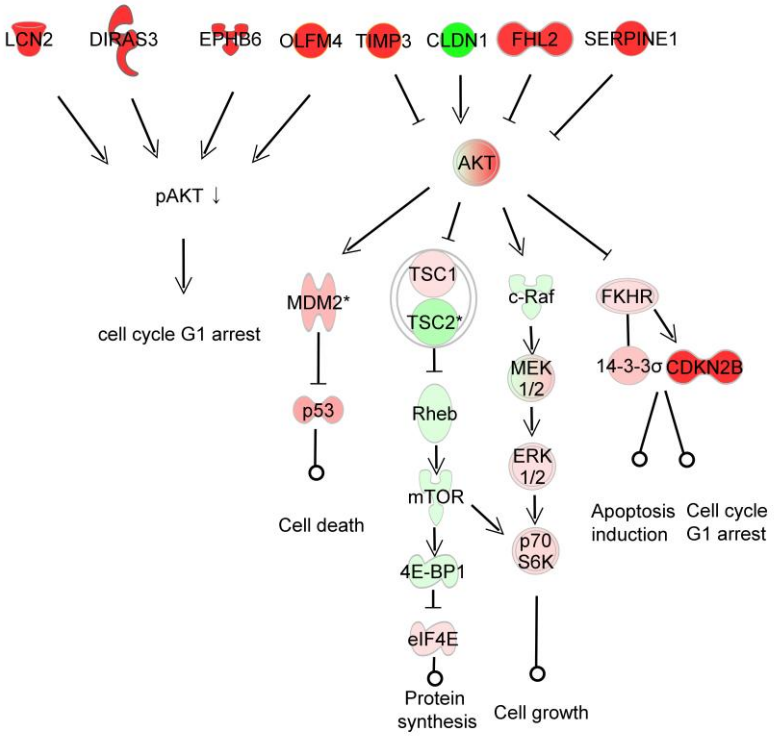
Table 4. Gene set analysis using Biocarta pathway database

Biocarta pathway	Pathway description	Number of genes	Adjusted q-value[24]
h_ifnaPathway	IFN alpha signaling pathway	10	0.023
h_il22bppathway	IL22 Soluble Receptor Signaling Pathway	11	0.023
h_aktPathway	AKT Signaling Pathway	26	0.023
h_ghPathway	Growth Hormone Signaling Pathway	32	0.023
h_ghrelinPathway	Ghrelin: Regulation of Food Intake and Energy Homeostasis	16	0.023
h_pcafpathway	The information-processing pathway at the IFN-beta enhancer	11	0.034
h_ifngPathway	IFN gamma signaling pathway	7	0.034
h_raccPathway	Ion Channels and Their Functional Role in Vascular Endothelium	15	0.034
h_ctlPathway	CTL mediated immune response against target cells	11	0.036
h_pmlPathway	Regulation of transcriptional activity by PML	25	0.036
h_reckPathway	Inhibition of Matrix Metalloproteinases	9	0.036
h_flumazenilPathway	Cardiac Protection Against ROS	5	0.036
h_tgfbPathway	TGF beta signaling pathway	23	0.039
h_achPathway	Role of nicotinic acetylcholine receptors in the regulation of apoptosis	13	0.041
h_alternativePathway	Alternative Complement Pathway	8	0.044
h_lympathway	Adhesion and Diapedesis of Lymphocytes	14	0.044
h_lymphocytePathway	Adhesion Molecules on Lymphocyte	11	0.045
h_nthiPathway	NFkB activation by Nontypeable Hemophilus influenzae	28	0.046
h_monocytePathway	Monocyte and its Surface Molecules	13	0.048

Table 5. Molecules targeting Akt signaling in differentially expressed genes between radiosensitive and radioresistant cell lines

Gene symbol	Role in cell	Up- or Down-regulation (Radiosensitive/Radioresistant)
DIRAS3	Apoptosis, a putative tumor suppresor gene	Up-regulated
CDKN2B	Cyclin-dependent protein kinase inhibitor activity	Up-regulated
LCN2	Suppression of proliferation and invasion	Up-regulated
OLFM4	Suppression of cell growth	Up-regulated
SERPINE1	Negative regulator of cell growth	Up-regulated
EPHB6	Transfection reduced *in vitro* invasiveness	Up-regulated
TIMP3	Apoptosis, inhibitors of the matrix metalloproteinases	Up-regulated
FHL2	Suppression of VEGF-induced PI3/Akt activity	Up-regulated
CLDN1	Activation of Wnt and PI3/Akt signaling	Down-regulated

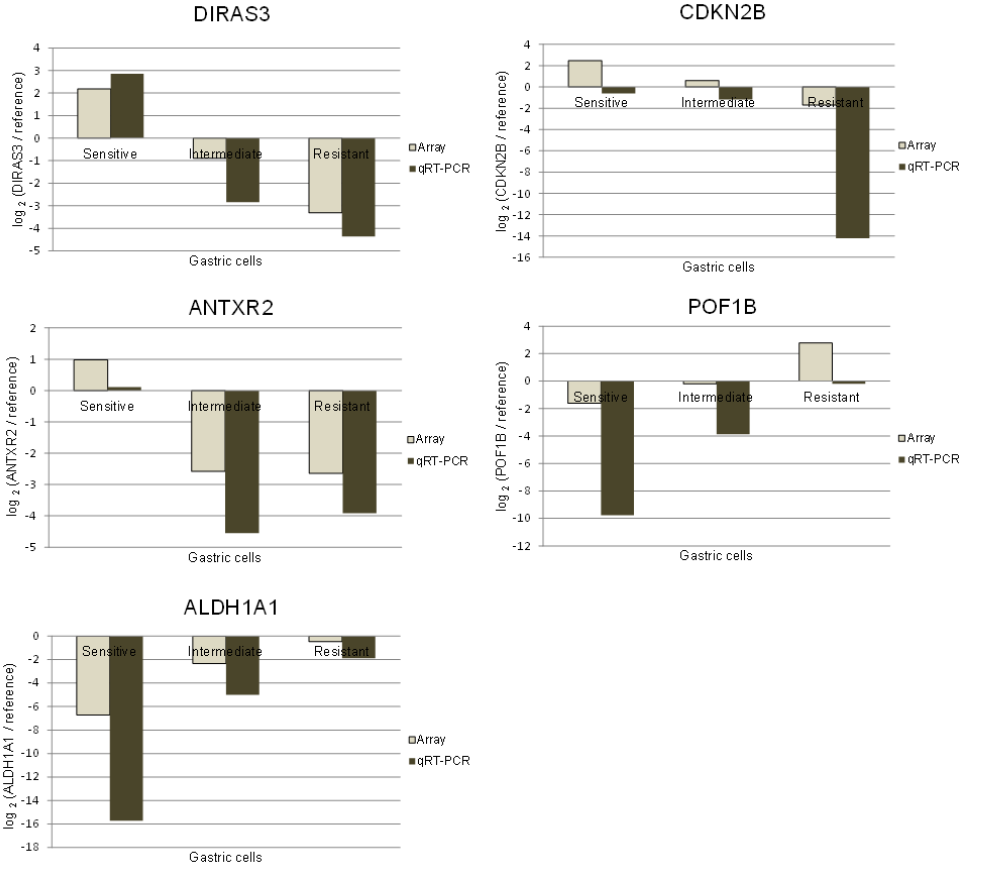
Figure 5. Akt signaling pathway with related genes of identified 68 gene signature. Red genes indicates up-regulation of gene expression in radiosenstivie cells and green genes indicates down-regulation.



Validation with quantitative real-time PCR validation

To validate the microarray results, we selected 5 genes (*DIRAS3*, *CDKN2B*, *POF1B*, *ALDH1A1*, *ANTXR2*) for qRT-PCR, which showed fold-changes more than 10-folds. *DIRAS3* and *CDKN2B* were down-regulated and *ALDH1A1*, *POF1B*, and *ANTXR2* were up-regulated in radiosensitive cells (Figure 6). The qRT-PCR results were well correlated with microarray results (the Pearson correlation coefficient ranging from 0.91 to 0.99).

Figure 6. Real-time PCR measurements and comparison with microarray-based gene expression levels



IV. DISCUSSION

To identify radiosensitivity signature genes and relevant signaling pathways in gastric cancer, we measured SF2 (radiosensitivity) and gene expression before radiation in twelve gastric cells. We selected 68 genes as radiosensitivity signature by comparing radiosensitive with radioresistant cells and these genes were related to several signaling molecules including *VEGF, AKT, TGF-β, NFκB, ERK, PI3K, HIF1A, MDM2, TGFB,* and *TP53* in genetic networks. In gene set analysis using entire genes, Akt signaling pathway was overrepresented and selected as potential druggable target regarding radiosensitivity. To validate microarray results, 5 genes were selected and validated by RT-PCR.

The combination of radiotherapy with targeted agents could overcome radioresistance with relatively less toxicity compared to cytotoxic agents[13]. In pathway analysis using 68-signature genes, we identified several targetable molecules including *PI3K, AKT,* and *ERK*. We summarized molecular target, function in radioresistance, and targeting agents in Table 6. It might be appreciated to test these molecular targets for radiotherapy enhancement.

Table 6. Molecular targets and targeting agents for radiosensitization

Molecular target	Function in radioresistance	Targeting agents
PI3K	Cell cycle progression, protection from apoptosis	PX-866, BKM120
AKT	Cell cycle progression, protection from apoptosis	MK-2206, perifosine
ERK	Activation of c-Myc and DNA-PKcs	U0126
P53	Mutation of p53 disturbs DNA repair and apoptosis	pifithrin-α
MDM2	Modulation of p53 pathway	nutlin-3
HIF1A	Transcription factor, resistance to oxidative stress	RX-0047
VEGF	Tumor angiogenesis	bevacizumab, ranibizumab
NFκB	DNA damage repair upon radiation	bortezomib, DHMEQ
TGF-β	DNA damage repair upon radiation	SB-431542, LY2109761

AKT is serine/threonine protein kinase and a major signaling molecule of phosphatidylinositol 3-kinase (PI3K)/AKT/mTOR pathway, which activates

downstream molecules involved in cell survival, cell cycle, and proliferation[25]. In gastric cancer, amplification of AKT1 and somatic mutation of AKT2 has been reported and about 80% of tumors harbors expression of AKT and phosphorylated AKT which showed statistically significant correlation with poor outcome.[26,27] AKT mediated radioresistance has been suggested via activation of DNA-dependent protein kinase catalytic subunit (DNA-PK_{cs}) which is a major enzyme of the DNA-double strand break repair[28], decreased degradation of cyclin D1 which is crucial for cell cycle progression[29], and inactivation of pro-apoptotic effector protein BAD[30]. Currently, several Akt inhibitors have shown the radiosensitive activity in lung cancer *in vitro*[31] and in prostate cancer *in vivo*.[32] In our study, AKT signaling pathway was overrepresented in gastric cancer cells which showed different expression between radiosensitive and radioresistant cells. As radiosensitizer, inhibiting PI3K/AKT/mTOR pathway or Akt inhibitor might be promising druggable target in gastric cancer.

We validated 5 of 68-signature genes whose microarray results were well correlated with qRT-PCR (*DIRAS3*, *CDKN2B*, *POF1B*, *ALDH1A1*, *ANTXR2*). *ALDH1A1* (aldehyde dehydrogenase 1 family, member A1) has been used for cancer stem cell marker and *ALDH1A1* expressing tumors harbor poor clinical outcomes in ovarian cancer and chemoresistance[33]. ALDH1A1 silencing sensitized to chemotherapy in ovarian cancer cells.[34] In our study, *ALDH1A1* is highly up-regulated in radioresistant cells and *silencing ALDH1A1 might* be associated with overcoming radioresistance. *DIRAS3* is a putative tumor suppressor gene which is expressed in normal ovarian and breast epithelium while not in ovarian and breast cancer.[35] Re-expression of *DIRAS3* showed an inactivation of the mTOR pathway in hepatocellular cancer[35] and chemosensitization to paclitaxel through G2/M cell cycle arrest in breast cancer cells[36]. In our study, *DIRAS3* expression is up-regulated in radiosensitive cells and might be associated radiosensitization when highly expressed. *CDKN2B*

encodes a cyclin-dependent kinase inhibitor and controls cell cycle G1 progression that one of targetable mechanism in radiosensitivity.[37] *ANTXR2* which binds to collagen IV and laminin is thought to have a role in extracellular matrix adhesion.[38] *POF1B* has function in actin binding and these adhesion-related molecules has been suggested to be important for radioresistance through interaction with the extracellular matrix.[39]

V. CONCLUSION

We first identified 68-radiosensitive gene signature in gastric cancer cells. Akt signaling pathway could be druggable target for radiosensitization in gastric cancer. We suggest that this analysis could elucidate targets for radiosensitivity biomarker discovery and the identified genes and signaling pathways could be served as potential targets in radiotherapy.

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ABSTRACT(IN KOREAN)

위암세포주의 방사선치료 효과 관련 유전자군의 탐색

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배경 : 방사선 치료에서 치료효과를 예측하는 것은 방사선치료 영역에서 중요한 과제이며 방사선 저항성을 극복하기 위한 표적을 발굴하고자 여러 연구가 진행되고 있다. 위암 세포주에서 방사선 감수성에 따라 발현에 차이가 나는 유전자군을 탐색하기 위해 본 연구를 수행하였다.

방법 : 마이크로어레이를 12종류의 위암 세포에서 방사선 조사전 시행하였다. 각 세포의 방사선 감수성을 측정하기 위해 2Gy의 방사선조사 후 집락형성분석법을 시행하였다. 방사선 감수성에 따른 유전자 발현분석을 시행하여 방사선 민감, 저항 세포에서 차이가 나는 유전자를 선별하고 유전자군 분석과 분자 경로 분석을 시행하였다.

결과 : 유전자 각각의 탐색을 통해 위암에서 방사선 민감도와 관련된 68개의 유전자를 탐색하였다. 탐색된 유전자는 분자 경로 분석을 통해 VEGF, AKT, TGF-β, NFκB, ERK, PI3K, HIF1A, MDM2, TGFB1, TP53와 관련이 있었다. 탐색된 유전자 네트워크는 세포 성장, 세포 유동성, 세포 주기와 관련이 있었다. 유전자군 분석을 통해 Akt 신호경로와 관련이 되어 있었다.

결론 : 위암 세포주에서 처음으로 방사선감수성과 관련된 68개의 유전자 군을 탐색하였다. Akt 신호전달 경로는 위암에서 방사선감수성과 관련된 가능성 있는 표적중의 하나였다. 본

연구의 분석방법은 향후 방사선 치료에서 표적 발굴에 응용될 수 있으리라 기대된다.

핵심되는 말 : 방사선감수성, 위암, 유전자 발현 분석,
집락형성분석법, Akt