CDC25A, VAV1, TP73, BRCA1 and ZAP70 gene overexpression correlates with radiation response in colorectal cancer

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Abstract. Radiotherapy is increasingly used in adjuvant approaches for colorectal cancer (CRC) to reduce local recurrence and improve survival. However, the principal limitation is the large variability in response among different individuals due to tumor heterogeneity. In the present study, we compared gene expression profiles between radiosensitive and radioresistant colorectal cancer cell lines to identify radiation-related molecules that can be used to evaluate the effects of radiation. The CRC cell line SW620 was irradiated with a high-energy photo beam. Following radiation treatment, RNA was extracted from non-irradiated and irradiated cells, respectively, and gene expression analysis was performed by oligonucleotide microarray and the DAVID bioinformatics method. To further confirm the results, an additional 4 CRC cell lines, COLO205, T84, HCT116, SW480 and SW403 were purchased from ATCC. The radiosensitivities of each were determined by the survival fraction at 2 Gray (SF2) of the surviving cells using the ATPLite assay, and the gene expression profiles after irradiation among the radiosensitive and radioresistant cell lines were analyzed by membrane arrays. The relationships between gene expression and patient clinicopathological features were also analyzed using membrane arrays and RT-PCR. The results from oligonucleotide microarray analysis show that 1601 genes were up-regulated (gene expression ratio of post- to pre-radiation treatment >2). By bioinformatic database analysis, 30 up-regulated genes were identified as involved in DNA damage response pathways, immune response pathways and the complement and coagulation cascades pathway. Fifteen genes showed differential gene expression profiles between radiosensitive (HCT116 and SW620) and radioresistant CRC cell lines (SW403 and SW480). In 110 CRC tissues, we detected five genes CDC25A, VAV1, TP73, BRCA1 and ZAP70 from 15 overexpressed genes that significantly related to prognostic factors (tumor size, advanced stage, invasive depth, lymph node metastasis and differentiation). These findings suggest that CDC25A, VAV1, TP73, BRCA1 and ZAP70 may be novel markers for predicting the effectiveness of radiotherapy in CRC patients.

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Introduction

Colorectal cancer (CRC) remains one of leading causes of cancer mortality worldwide. Preoperative radiotherapy (RT) has been widely used as a major treatment modality to improve local control, as well as to improve survival (1-3). However, response to radiotherapy differs among individual tumors. In radiation unresponsive cases, there may have disadvantages such as delaying necessary surgery or immune suppression. Although many clinical factors (4,5), radiological findings (6,7), and molecular markers (8,9) have been suggested to be related to the therapeutic response of CRC, the clinical usefulness of them to predict therapeutic response remains controversial. There are currently no reliable ways of identifying the individuals who may respond well to radiation. Thus, to identify factors that can be applied to predict RT efficacy before treatment will aid in making appropriate treatment strategy decisions.

Because cellular responses rely in part on changes in gene expression, the extent to which the radiation-responsive genes are induced or repressed influences how cells deal with radiation exposure, where individual variability of radiation sensitivity is observed at the gene expression level (10,11). In

2000, Lehnert *et al* reported that gene chips could be used to survey radiation-associated genes (12). Physicians explored different gene expression patterns between radiosensitive and radioresistant tumors in uterine cervical cancer (13), and to predict the clinical RT response in esophageal cancer (14) by microarray methods. However, the study of CRC and radiation-associated gene expression is still limited. Recently, there were two studies to identify the clinical preoperative RT response of rectal cancer, and was reported that tumor responsiveness was associated by gene profiling by microarrays (54 genes found by Ghadimi *et al* and 54 genes found by Watanabe *et al*) (15,16). But such large numbers of gene profiles are difficult for physicians to effectively apply in clinical treatment decisions.

In 2004, Dairkee *et al* demonstrated that the gene expression of primary breast cancer cell cultures resembled tumor tissue (17). In the present study herein, we examined radiosensitivity in CRC cell lines to identify a set of discriminating genes that can be used for prediction of response to RT in human CRC. This laboratory utilized the high efficiency gene screening technology of microarrays to analyze the gene expression of irradiated CRC cells. From these tests we sought genes with radiation-related high expression that may serve as responsive markers. Therefore, we used paired irradiated and unirradiated SW620 cells (Dukes' C colorectal adenocarcinoma) and performed microarray analysis using the Agilent Oligo array. As a result, 1601 genes with expression levels in irradiated cells 1.5-fold higher than unirradiated cells were selected. We discovered 30 radiation-pathway related genes by employing bioinformatics studies. Using a membrane array, 15 out of 30 genes have more significant differences between radiosensitive and radioresistant cells. Results from this study demonstrated that five genes CDC25A, VAV1, TP73, BRCA1 and ZAP70 from 15 overexpressed genes were significantly related to clinicopathological factors (tumor size, advanced stage, invasive depth, lymph node metastasis and differentiation) which correlated to cancer prognosis in 110 stage I-IV CRC patients. These five genes might be potential new markers for radiation response prediction. Information of this nature is also of benefit in the selection of treatment options, as well as the assessment of individual response.

Materials and methods

ATCC cell lines and radiation. The cell lines used in this study were COLO205, HCT116, SW480, SW620 and SW403, five human CRC cell lines, which were obtained from ATCC (American Type Culture Collection, ATCC, Rockville, MD, USA). SW480, SW620 and SW403 cells were cultured in Leibovitz's L-15 medium, supplemented with 10% fetal bovine serum (FBS) and glutamine. COLO205 was cultured in RPMI-1640 medium supplemented with 5% FBS and sodium pyruvate. HCT116 cells were cultured in McCoy's 5a medium supplemented with glutamine and 10% FBS. All medium were added 1% antibiotic-antimycotic of cell cultivating solution and incubated at 37˚C and 100% air. Cells were irradiated at room temperature, using a Medical LINAC (medical Linear accelerator). The energy of the photon beam was 6 MeV with the dose rate of 400 mu/min. The total dose of radiation was 2, 4, 6 or 8 Gy to all CRC cell lines for radiation sensitivity analysis. The total dose of radiation was 16 Gy to SW620 cells for further microarray analysis of radiation-induced overexpression of genes.

ATPLite luminescence ATP detection assay for cell viability assessment. The radiosensitivities of cells were determined by survival fraction at 2 Gray (SF2) of the surviving cells (18). In the present study, the ATPLite assay (Packard Instrument Co., USA) was used to quantify viable cell density of the sample (19). Cells were harvested from exponential phase cultures by trypsinization, counted and then plated into 48-well plates. Seeding densities were 10^3 cells per well for each of the cell lines. Cells were incubated for 6 double times after radiation over the dose range 0-8 Gy. Subsequently 25 μl of cell lysis buffer solution was added into each well and the substrate solution (luciferase/luciferin). ATPLite luminescence was measured in a TopCount Microplate reader (Packard Instrument Co.). The percentages of living cells were calculated per well. Results of SF2 from 6 replicate assays per cell line were analyzed.

Patients and tissue specimens. Enrolled in this study were 110 American Joint Commission on Cancer/International Union Against Cancer (AJCC/UICC) stage I-IV CRC patients (mean age, 62.07±10.85 years) who underwent surgical treatment in the Department of Surgery at Kaohsiung Medical University Hospital, Taiwan. Patients with other malignant diseases in their medical history were excluded. All 110 patients underwent radical resection for a primary lesion. The data of these 110 CRC patients were used for analysis of radiation pathway-related gene expressions and the correlation between gene expression profiles and patient clinicopathological features. All paired samples including tumor and tumor-free tissues were obtained from all patients. Tumor grading was carried out and confirmed by pathologists. Each tissue sample was snap-frozen in liquid nitrogen immediately after surgery or biopsy, and stored at -80˚C. Samples were further used in experiments for real-time PCR, membrane array analysis, and immunoblotting. Clinical stage and pathological features of primary tumors were defined according to criteria of the AJCC/UICC (20). Written informed consent was obtained from all subjects and/or guardians for the use of patient tissue samples. Tissue acquisition and subsequent use were approved by the institutional review board of Kaohsiung Medical University Hospital. Complete medical history, physical examination, and laboratory studies, including assessing serum carcinoembryonic antigen (CEA) levels were reviewed. Computed tomography (CT) or magnetic resonance imaging (MRI) of abdomen, abdominal ultrasonography, and chest radiography, bone scans, and colonoscopy were performed before surgical intervention.

Total RNA extraction and first strand cDNA synthesis. Total RNA was isolated from each CRC patient's tissue and from cell lines with ISOGEN™ (Nippon Gene, Toyama, Japan) and the QIAmp® Mini Kit (Qiagen Inc., Valencia, CA) according to the manufacturer's instructions (21). RNA purified is quantified by OD 260 nm by an ND-1000 spectrophotometer (Nanodrop Technology, USA) and quantitated by Bioanalyzer 2100 (Agilent Technology, Palo Alto, CA, USA). First strand

cDNA was synthesized from total RNA by using a RT-PCR Kit (Promega Co., Madison, WI). The reverse transcription was carried out in a reaction mixture consisting of a 25 μg/ml oligo (dT) 15-mer primer, 100 mmol/l PCR Nucleotide Mix, 200 μmol/l M-MLV Reverse Transcriptase, and 25 μl of Recombinant RNasin® Ribonuclease Inhibitor (Promega). The reaction mixtures with RNA were incubated at 42˚C for 2 h minimum, heated to 95˚C for 5 min, and then stored at -80˚C until analysis.

Oligonucleotide microarray analysis. The oligonucleotide array contains 22,500 elements designed for expression profiling (Human 1A V2, Agilent Technologies), for which over 18,000 well-characterized, full-length human genes have been defined. First-strand cDNA targets for hybridization were made by reverse transcription of the mRNA isolated from both the unirradiated and 12, 24, 48, 72 h after ionizing radiation (total dose 16 Gy) from SW620 cells by using SuperScript II RT (Gibco-BRL, Gaithersburg, MD, USA) in the presence of either Cy3- or Cy5-labeled dUTP (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The targets were dried to 18 μl by a SpeedVac™ concentrator (Thermo Electron Co., Waltham, MA, USA), and 3.6 μl 20X SSC, 1.8 μl 10 mg/ml poly-A and 0.54 μl 10% SDS were added. Then, the mixture was heated to 100˚C for 2 min proceeding to the hybridization reaction on Human 1A Oligo Microarray V2 array slides (Agilent Technologies) in an incubator at 60˚C for 17 h. After being sequentially washed with 1X SSC, 0.2X SSC and 0.5X SSC, hybridized microarray slides were scanned and fluorescence signals were detected by using an Axon GenePix Pro 3.0™ (Axon Instruments). The acceptance criterion for a gene signal was a signal-to-noise ratio of ≥2. If either the Cy3 or Cy5 signal of a specific spot passed the criterion, the flag of its ratio was counted to be 'True'. The element with the 'True' flag was analyzed with GeneSpring GX7 (Silicon Genetics, Redwood City, CA, USA). The differentially expressed elements were analyzed by the two-sided statistical tolerance interval (95%).

Real-time polymerase chain reaction (RT-PCR). For each reaction, 2.0 μl of each cDNA sample were used. Sequences of the 30 oligonucleotide primers were designed according a PCR primer selection program based on primer 3 at http://frodo.wi.mit.edu/cgi-bin/primer3/prime3www.cgi (Table I). Each RT-PCR reaction mixture contained 1X PCR buffer (10 mmol/l Tris-HCl, pH 8.3, 50 mmol/l KCl, 2 mmol/l $MgCl_2$), 50 μmol/l dNTP, 0.1 μmol/l sense and antisense primers for target genes, and 0.1 μmol/l sense and antisense primers for β-actin. PCR products were analyzed on 3% agarose gel containing 0.5 μg/ml ethidium bromide. The signals on the UV transilluminator for each target gene and β-actin expression levels were scanned with a computing laser densitometer (Alpha Inotech, San Leandro, CA) to calculate the reactive mRNA density. Real-time PCR was performed in a Rotor-Gene 2000 thermocycler (Corbett Research, Inc.). PCR products (i.e. synthesized dsDNA) were quantified by measuring the fluorescent intensity at the end of each amplification cycle. For each sample, real-time PCR analysis was repeated in three independent experiments to ensure the reproducibility of results. We used β-actin and *Oryza sativa* sequence individual to be positive and negative controls, respectively, and used dd water to be a blank control.

Membrane array. The procedure of the membrane-array method for gene detection was performed based on our previous work (22). Visual OMP3 (Oligonucleotide Modeling Platform, DNA Software, Ann Arbor, MI) was used to design probes for target genes and β-actin, and the latter served as an internal control (Table I). The newly synthesized oligonucleotide fragments were dissolved in distilled water to a concentration of 20 mM, applied to a BioJet Plus 3000 nl dispensing system (BioDot Inc., Irvine, CA, USA), which blotted the target oligonucleotide, and the β-actin control sequentially (0.05 μl per spot and 1.5 mm between spots) on SuPerCharge nylon membrane (Schleicher and Schuell, Dassel, Germany) in triplicate. DMSO was also dispensed onto the membrane as a blank control (Fig. 1). After rapid drying and cross-linking procedures, the preparation of membrane array was accomplished. The membrane array was used to analyze the gene expression among the radiosensitive and radioresistant CRC cell lines (Fig. 2); and to compare gene expression of tumor and normal counterpart tissue in 110 CRC patients.

Preparation of digoxigenin (DIG)-labeled cDNA targets and hybridization. First-strand cDNA targets for hybridization were made by reverse transcription of the mRNA from the tumor and corresponding normal tissues of CRC patients in the presence of DIG-labeled UTP (Roche Diagnostics GmbH, Penzberg, Germany) using SuperScript II reverse transcriptase (Gibco-BRL). The hybridized arrays were then scanned with an Epson Perfection 1670 flatbed scanner (SEIKO Epson Corp., Nagano-ken, Japan). Subsequent quantification analysis of each spot's intensity was carried out using AlphaEase® FC software (Alpha Innotech Corp.). Spots consistently carrying a factor of two or more were considered as differentially expressed. A deformable template extracted the gene spots and quantified their expression levels by determining the integrated intensity of each spot after background subtraction. The fold ratio for each gene was calculated as follows: spot intensity ratio = mean intensity of target gene/mean intensity of β-actin. Fig. 1 provides the schematic representation of the membrane array with 30 target genes, one housekeeping gene (β-actin), one bacterial gene (TB), and the blank control (dd water).

Statistical analysis. All data were analyzed by using the statistical Package for the Social Sciences Version 12.0 software (SPSS Inc.). The correlation between SF2 of five CRC cell lines was analyzed by one-way ANOVA. Gene expression profiles between radiosensitivity and radioresistant cell lines were analyzed by the Independent t-test. The correlation between the clinicopathological features and gene expressions in CRC patients was compared using the χ^2 test. $P<0.05$ was considered statistically significant.

Results

Microarray analysis of colorectal cell lines. SW620 cells were irradiated with 16 Gy to induce sublethal DNA damage

Table I. Oligosequences of 30 target genes which related to the radiation associated genetic pathway by BIOCATA and KEGG database analysis after microarray analysis.

Gene name	Oligonucleotide sequence
Complement and coagulation cascades	
CFI	TGCACAGCCAACTTCATCTTCCCCTGTAATGCAGTCCACCTCACCATT
PTGS1	ATAGCCCAACCAAACTGACAAATAAAACCAACCATCACATCCACCAAATC
CFB	TCCTTCAGCCAGGGCAGCACTTGAAAGAGGTTGATGTGAAAGTCTCG
PROCR	CCCTATTATATCAGCGTCCCATCCCAAGTCTGACACACCTGGAAGTATTG
FGA	ATGTGCCTAAATCCATTGCCTCGGGACAGTCAGAACCATCTTCGGAG
VWF	CCTGCACATCGTTGATGTCAATGGAGTACATGGCTTTGCTGGCACATT
F2	CCAAAGGTCCTCGGATTGAAGAAAGTCTGGTACTCACTGGTGGCGGTACG
FGG	TGGTGTTGCTGTCCTTCTCCAATTGTGAGTCTGTTGAATGGGATTATCTT
PLG	CACAATCAAATGAAGGGGCCGCACACTGAGGGACATCACAGTAGTCGTAA
DbpB (YBX1)	GGACCTGTAACATTTGCTGCCTCCGCACCCTTTTCTCCTTCAACAACATC
Immunization	
ANPEP	CATACTCGGTGGAGAATCGTCGTGTCACTGCCTGGATGAGGTTGGA
CXCR4	GAAACTGGAACACAACCACCCACAAGTCATTGGGGTAGAAGCGGTCA
PTPN7	TCAGGGAGCAGAGTCACTGATAGGATGTTGAGACTTGGAGATCAGGACCA
TLR2	TCAATGATATTGTCAATGATCCACTTGCCAGGAATGAAGTCCCGCTTAT
CD33	AATCTCCAAGAATCAGCCTTTGGTCCCCGACCTGTAGAGGATGTGGATCT
RHOH	AGGGCAAGTTGCTCCTAATTTCACCAATCCACTTGTTCTTCAAGTTCAGG
ZAp70	TCAAGAGGGAAGCTGGGTTTATTCTGGTGATCTACGCCTGACCACCTGA
IL8	AATTCTCAGCCCTCTTCAAAAACTTCTCCACAACCCTCTGCACCCAG
VAV1	TAGTAGATGTATTTCTGCCCTCCCACCAGTTCTGTTCAGCCTCAGCCTTC
CD59	GCATGTAAGGTTAAGTAACTTGCCTGAGGGTCGCCGATTAGCATCAGAGC
ICAM1	CCCATTATGACTGCGGCTGCTACCACAGTGATGATGACAATCTCATACCG
DNA damage response	
CASP1	CTACCATCTGGCTGCTCAAATGAAAATCGAACCTTGCGGAAAATTTCCTC
TP73	TGGTAAATGCTCTGTAACCCTTGGGAGGTGAAATACTCGATGCAGTTTGG
TGFB2	CTTTCACCAAATTGGAAGCATTCTTCTCCATTGCTGAGACGTCAAATCGA
SMC1B	CCACACACTTTTCTCCCCCTGACAAATTGTCCATTGGCATAAACCGTT
BRCA1	TCATTTCTAATACCTGCCTCAGAATTTCCTCCCCAATGTTCCACTCCAAC
CDC25A	ACCAAGCTCCCTCCTGAGAAAATTCTATCCAGTCATGTCCATGCCAGCAC
RAD51	CTGAAAGCTCACCTCGACCCGAGTAGTCTGTTCTGTAAAGGGCGGTGG
ESPL1	TCACATCCCAGAGATTACCCAGAAACAAGGGGCAACCAGCCATGATGTAC
CDKN1A (p21)	GTACACTAAGCACTTCAGTGCCTCCAGGGGCTCAACGTTAGTGCCAGGAA

CFI, complement factor I; PTGS1, prostaglandin-endoperoxide synthase 1; CFB, complement factor B; PROCR, protein C receptor, endothelial; FGA, fibrinogen α chain, transcript variant α; VWF, von Willebrand factor; F2, coagulation factor II (thrombin); FGG, fibrinogen γ chain, transcript variant γ-A; PLG, plasminogen, mRNA; dbpB, DNA-binding protein B; ANPEP, alanyl (membrane) aminopeptidase; CXCR4, chemokine (C-X-C motif) receptor 4; PTPN7, protein tyrosine phsophatase, non-receptor type 7; TLR2, toll-like receptor 2; CD33, CD33 molecule; RHOH, ras homolog gene family, member H; ZAp70, ζ-chain (TCR) associated protein kinase 70 kDa; IL8, interleukin 8; VAV1, vav 1 guanine nucleotide exchange factor; CD59, CD59 molecule, complemetnt regulatory protein, transcript variant 1; ICAM1, intercellular adhesion molecule 1, human rhinovirus receptor; CASP1, caspase-1, apoptosis-related cysteine peptidase; TP73, tumor protein p73; TGFB2, transforming growth factor β-2 precursor; SMC1B, structural maintenance of chromosomes 1B; BRCA1, breast cancer 1; CDC25A, cell division cycle 25 homolog A; RAD51, RAD51 homolog; ESPL1, extra spindle pole bodies homolog 1; CDKN1A (p21), cyclin-dependent kinase inhibitor 1A.

and were incubated at four time points (12, 24, 48 and 72 h, respectively). We utilized four groups of cells with different incubation durations and paired them with cells without radiation. All results from the experiments underwent standardized analysis and validation. Then, we used GeneSpring Biological data analysis software, where hierarchical clustering was performed in the experimental groups to initially assess gene expression of all genes on the chip. After further analysis and validation, 1601 genes displayed overexpression and potentially served as biological markers.

VWF	F2	FGG	FGA	PLG	dbpB	PROCR	PTGS1	CFI	CFB	Blank	β-actin
VWF	F2	FGG	FGA	PLG	dbpB	PROCR	PTGS1	CFI	CFB	Blank	β-actin
VWF	F2	FGG	FGA	PLG	dbpB	PROCR	PTGS1	CFI	CFB	Blank	β-actin
ANPEP	CD33	TLR2	ZAp70	IL8	CXCR4	VAV1	RHOH	ICAM1	PTPN7	CD59	Blank
ANPEP	CD33	TLR2	ZAp70	IL8	CXCR4	VAV1	RHOH	ICAM1	PTPN7	CD59	Blank
ANPEP	CD33	TLR2	ZAp70	IL8	CXCR4	VAV1	RHOH	ICAM1	PTPN7	CD59	Blank
TGFB2	P21	CDC25A	SMC1B	ESPL1	RAD51	BRCA1	CASP1	TP73	Blank	Blank	β-actin
TGFB2	P21	CDC25A	SMC1B	ESPL1	RAD51	BRCA1	CASP1	TP73	Blank	Blank	β-actin
TGFB2	P21	CDC25A	SMC1B	ESPL1	RAD51	BRCA1	CASP1	TP73	Blank	Blank	β-actin

Figure 1. Location of dotting on gene array nylon membranes. The arrangement of gene dotting on the nylon membranes is demonstrated. Each tested gene was dotted three repeated times on the nylon membrane. On the right side, three repeated dots of negative control (TB, tuberculosis gene); three repeated dots of positive control (β-actin) and three repeated dots of blanks (50% DMSO).

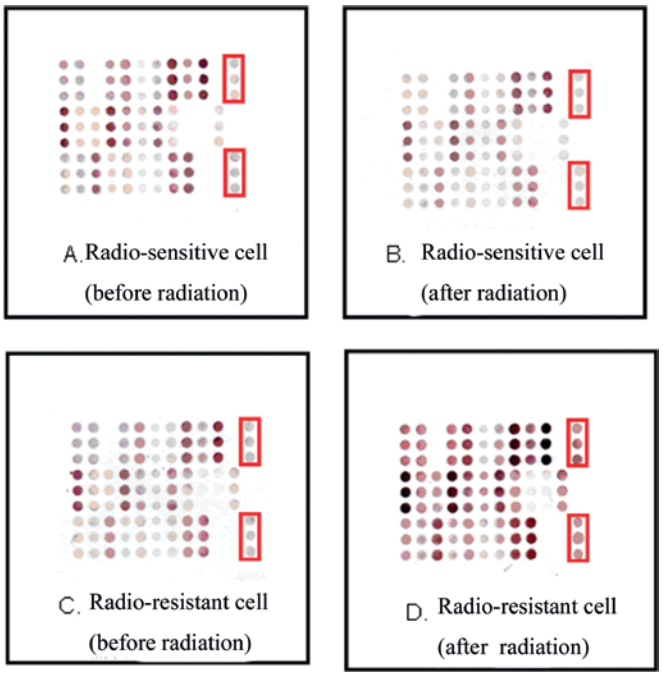

Figure 2. Results of color expression of Nylon membrane gene array. Color effects of the 30 target genes on nylon membranes are shown. (A and B) The genetic expression of unirradiated and irradiated HCT116 cell lines. (C and D) The genetic expression of unirradiated and irradiated SW403 cell lines.

Identification of candidate genes by bioinformatics and ATCC cell lines. The 1601 overexpressed gene validated in the SW620 cells, were analyzed with DAVID (Database for Annotation, Visualization, and Integrated Discovery) (23), and KEGG (Kyoto Encyclopedia of Genes and Genomes) databases (24), and results indicated that a total of 30 genes, and 3 identified pathways were enriched for genes associated with radiation response, including the DNA damage response, immunization and complement and the coagulation cascade. The constructed radiation-associated genetic pathway is shown in Fig. 3.

Radiosensitivity of colorectal cancer cell lines. The doubling time of five CRC cell lines, SW480, SW403, COLO 205, SW620 and HCT116, were examined and data are shown in Table II. SW403 has the longest doubling time, and HCT116 displayed the shortest doubling time. The SF2 of each cell line is shown in Table II and exhibited statistical significance (P=0.0034). The SF2 values of SW403, SW480 and COLO 205 cell lines were significantly larger than SW620 and HCT116 cell lines. Therefore, SW620 and HCT116 cell lines were categorized to be radiosensitive cell lines, and SW403, SW480 and COLO 205 cell lines were identified as radioresistant cell lines.

Differential gene expression between radiosensitive and radioresistant cell lines. To investigate the expression profiles of the afore-mentioned 30 genes in the four CRC cell lines with different radiation sensitivity, membrane array was used to detect the 30 gene expression levels from SW620, HCT116, SW403 and SW480 cells. The differential gene expression profiles of four CRC cell lines are shown in Table III. Using statistical independent t-test analysis, we found that there were

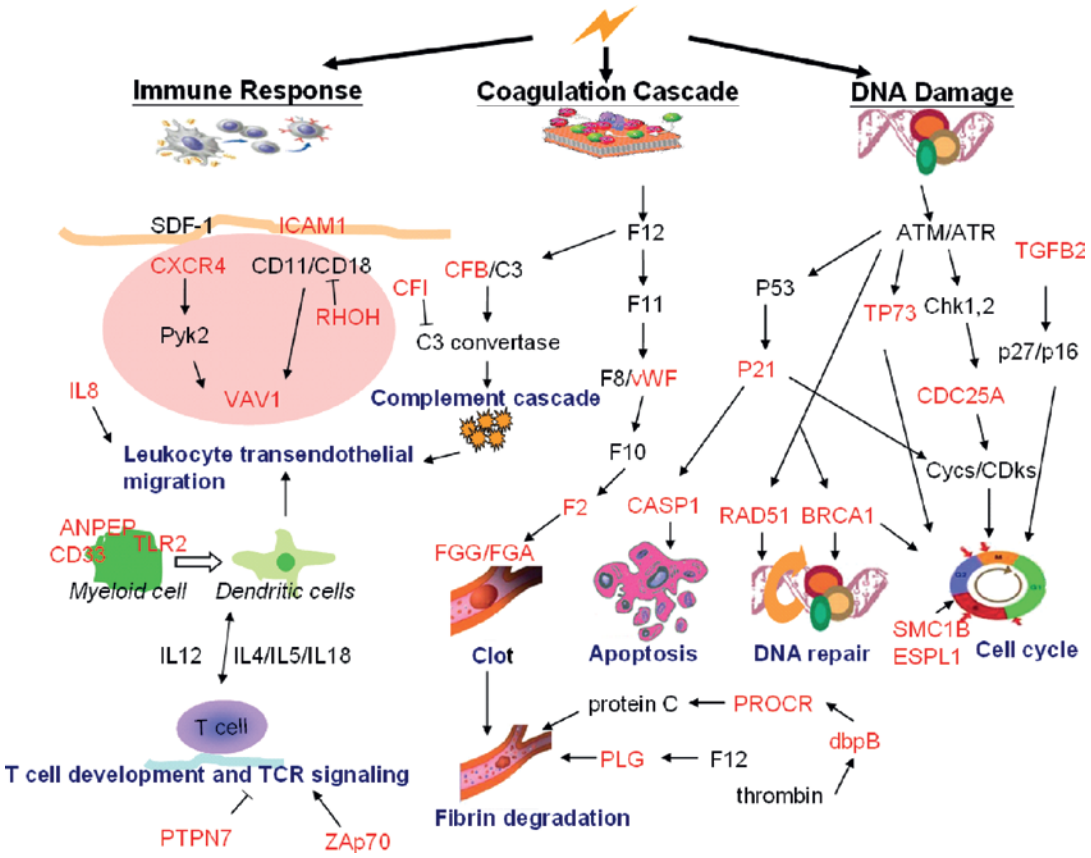

Figure 3. Radiation related pathways. There were three radiation related pathways including immune response, coagulation cascade and DNA damage pathways.

Table II. The doubling times and survival fraction at 2 Gray (SF2) of six colorectal cell lines.

Cell lines	Disease	Stage (Dukes')	Doubling time (h) (mean ± SD)	6 doubling time (day)	Measure time (day)	SF2 (mean ± SD) %
SW480	Colorectal adenocarcinoma	B	30.65±1.29	7.6	8	59.17±0.59
SW403	Colorectal adenocarcinoma	C	71.47±9.12	17.86	18	58.97±1.37
COLO205	Colorectal adenocarcinoma	D	31.45±2.69	7.86	8	54.16±10.56
SW620	Colorectal adenocarcinoma	C	28.92±5.62	7.23	8	32.55±5.14
HCT116	Colorectal adenocarcinoma	ND[a]	16.23±0.84	4.05	5	24.88±0.12

[a]ND, no data.

15 out of 30 genes with significant differential expression between radiosensitive and radioresistant CRC cells.

Gene validation in clinical colorectal cancer tissues by membrane array and RT-PCR. The afore-mentioned 15 genes which had significant differential expression between radiosensitive and radioresistant CRC cells, were validated in the colorectal tissues of clinical stage I-IV CRC patients, both membrane array and RT-PCR were conducted to detect the mRNA expression level from the 110 paired tissues. Based on the results of membrane array hybridization analysis, if the gene presented a color response of >2-fold between the CRC cancer and normal tissue, it was defined as overexpression. As shown in Table IV, we compared the correlation between gene expression and clinicopathological features of 110 CRC patients. Statistical analysis indicated that five genes, CDC25A, VAV1, TP73, BRCA1 and ZAP70, have a statistically significantly correlation to prognostic factors (including tumor size, advanced stage, invasive depth, lymph node metastasis and differentiation) (P<0.05).

Discussion

Preoperative radiotherapy has been widely used to improve local control of disease and to improve survival in the treatment of rectal cancer. However, there is a wide spectrum

Table III. Differential gene expression between radiosensitive and radioresistant cell lines.

Pathway/genes	HCT116 vs. SW480	HCT116 vs. SW403	SW620 vs. SW403
Complement and coagulation cascades			
CFI	–	–	–
PTGS1	[a]	[a]	–
CFB	[a]	[a]	–
PROCR	–	–	–
FGA	–	–	–
VWF	–	–	–
F2	–	–	–
FGG	–	–	–
PLG	–	–	–
DbpB	–	–	–
Immunization			
ANPEP	[a]	–	–
CXCR4	[a]	[a]	–
PTPN7	–	–	–
TLR2	–	–	–
CD33	–	–	–
RHOH	–	–	–
ZAp70	[a]	[a]	[a]
IL8	[a]	[a]	–
VAV1	[a]	[a]	[a]
CD59	[a]	[a]	–
ICAM1	[a]	[a]	–
DNA damage response			
CASP1	[a]	[a]	–
TP73	[a]	[a]	[a]
TGFB2	[a]	–	–
SMC1B	–	–	–
BRCA1	[a]	[a]	[a]
CDC25A	[a]	[a]	[a]
RAD51	–	[a]	–
ESPL1	–	–	–
p21	–	–	–

[a]Gene carried differential gene expression profiles between two cell lines with statistical significance.

of tumor responsiveness of rectal cancer to preoperative radiotherapy ranging from complete response to complete resistance. Pretreatment tumor intrinsic radiosensitivity is an important prognostic parameter, and contributes to prognosis independently of other established and putative parameters (25). In past studies, several genes were found to associate with radiation sensitivity, such as p53 (26-30), ras (31), raf-1 (32), and bcl2 (29,30), and they were related to DNA repair, apoptosis, cell cycle, growth factors, signal transduction and cell adhesion. In spite of some studies reporting radiation sensitivity associated with a gene in CRC, they were largely focused on single genes (26,33). A few studies have conducted comprehensive gene expression profiles by microarray analysis, but the mechanism of radiation sensitivity was still unclear (15,16,34).

SF2 was reported to be independent of prognostic factors of disease stage, tumor grade, patient age, colony-forming efficiency and tumor diameter in cancers (25). In the present study herein, we applied SF2 to classify CRC cell lines to be either radiosensitive or radioresistant groups. We began with gene expression response to radiation because cellular responses rely on changes on gene expression. Irradiation of cells may not only lead to cell death but to other changes as well. Many factors affect radiation response including the position of tumor cells within the cell cycle, which may confer radiosensitivity or resistance. For instance, the late G2 and M phases are the most radiosensitive, whereas the late S phase is the most radioresistant (35,36). Bioinformatics resources provide a comprehensive set of functional annotation tools for investigators to understand biological meaning behind large lists of genes. Microarray results of irradiated CRC cells were analyzed by DAVID (23), and KEGG (24) databases and the data identified 30 unregulated genes within DNA damage response, immunization and complement and coagulation cascade pathways. By determining 30 radiation-associated gene expression profiles using membrane array, we identified 15 novel discriminating genes of which the expression differed significantly between radiosensitive and radioresistant cell lines. The list of discriminating genes included PTGS1, CFB, ANPEP, CXCR4, ZAp70, IL8, VAV1, ICAM1, CD59, CASP1, TP73, BRCA1, CDC25A, RAD51 and TGFB2. In 2007, Knoops *et al* firstly reported irradiation-induced genes related to macrophage activation and immune response in follicular lymphoma patients (37). According to analysis of the complement and coagulation cascades pathway, previous studies have linked malignant transformation, tumor angiogenesis and metastasis to the generation of clotting intermediates (e.g. tissue factor, factor Xa and thrombin), clotting or platelet function inhibitors (e.g. COX-2), or fibrinolysis inhibitors (e.g. plasminogen activator inhibitor, type 1) (38-40); and an *in vitro* study used low frequency electrical fields to induce up-regulated genes related to hemostasis by microarray (41).

After validation in 110 clinical stage I to IV CRC patient tissues, CDC25A, VAV1, TP73, BRCA1 and ZAP70 were selected from 15 radiation-related genes to be the prognosis-related markers depending on their significant correlations to clinical prognostic variables. CDC25A, TP73 and BRCA1 are associated with the pathway of DNA damage response; ZAP70 and VAV1 are associated with the immune-related pathway. The five genes have also been reported to be prognostic factors in other cancers.

CDC25A was overexpressed in various human malignancies including non-Hodgkin's lymphoma, non-small cell lung cancer, and esophageal cancer (42-44), and also overexpressed in axoxymethane-induced murine colon cancer (45). CDC25A, CDC25B and CDC25C belong to a family of protein phosphatases which activate the cyclin-dependent kinases at different points of the cell cycle (46). Disorders of the cell cycle and cell cycle-regulating molecules are characteristics of cancer cells. Some of these disorders greatly affect the clinical outcome, independently of other

Table IV. Correlation of 15 gene overexpression and the clinicopathological features in 110 colorectal cancer patients (gene expression >2, positive).

		No. of cases with gene overexpression (%)														
	No.	PTGS1	CFB	ANPEP	CXCR4	ZAp70	IL8	VAV1	ICAM1	CD59	CASP1	TP73	BRCA1	CAC25A	RAD51	TGFB2
		52 (47.3)	38 (34.5)	52 (47.3)	68 (61.8)	66 (60)	64 (58.2)	61 (55.5)	53 (48.2)	61 (55.5)	60 (54.5)	65 (59.1)	66 (60)	66 (60)	61 (55.5)	47 (49.1)
Gender																
Male/Female	(71/39)	35/17	22/16	31/21	41/27	42/24	40/24	36/25	34/19	36/25	36/24	39/26	40/26	40/26	37/24	28/19
P-value		0.566	0.289	0.306	0.236	0.807	0.597	0.176	0.934	0.176	0.275	0.231	0.290	0.290	0.341	0.347
Age																
<60/≥60	(45/65)	23/29	15/23	22/30	26/42	25/41	24/40	21/40	23/30	25/38	20/40	22/43	21/25	28/38	25/36	18/29
P-value		0.502	0.824	0.778	0.468	0.429	0.391	0.123	0.609	0.762	0.077	0.070	0.391	0.692	0.986	0.630
Tumor size (cm)																
<5/≥5	(53/57)	27/25	17/21	21/31	32/36	31/35	29/35	31/30	23/30	25/36	25/35	24/41	28/38	30/36	27/34	25/22
P-value		0.457	0.599	0.121	0.764	0.755	0.477	0.537	0.333	0.092	0.134	0.005[a]	0.139	0.483	0.359	0.364
Stage[a]																
I+II/ III+IV	(50/60)	24/28	20/18	19/33	28/40	34/32	27/37	22/39	22/31	25/36	23/37	25/40	26/40	37/29	29/35	22/25
P-value		0.889	0.272	0.075	0.252	0.118	0.417	0.027[a]	0.423	0.293	0.1	0.077	0.118	0.006[a]	0.506	0.805
Invasion depth																
T1+T2/T3+T4	(47/63)	23/29	17/21	18/34	26/42	29/37	31/33	23/38	21/32	24/37	25/35	24/41	18/48	30/36	24/37	24/23
P value		0.763	0.757	0.103	0.226	0.753	0.153	0.235	0.526	0.424	0.805	0.139	<0.0001[a]	0.479	0.424	0.127
Lymph node metastasis																
(-)/(+)	(49/61)	23/29	20/18	19/33	28/40	33/33	27/37	21/40	22/31	24/37	22/38	24/41	25/41	36/30	25/36	21/26
P-value		0.950	0.215	0.110	0.366	0.159	0.557	0.017[a]	0.537	0.221	0.069	0.053	0.085	0.010[a]	0.402	0.980
Histology																
WD+MD/PD	(101/9)	49/3	25/3	47/5	63/5	64/2	57/7	57/4	47/6	58/3	26/4	50/4	52/3	47/5	43/4	39/2
P-value		0.382	0.936	0.603	0.687	0.016[a]	0.214	0.488	0.247	0.163	0.227	0.771	0.297	0.603	0.913	0.330

[a]International Union Against Cancer.

clinicopathological parameters, and have been found to be associated with sensitivity for radiotherapy via induction of tumor cell apoptosis (47). In 2000, Miyata *et al* found that CDC25B overexpression was associated with a high sensitivity for radiation therapy in human esophageal cancer (48). Postoperative survival was found poorer for CDC25A-positive esophageal squamous cell carcinoma patients (44). In the present study, CDC25A overexpression is more strongly associated with CRC patients with earlier cancer stage or without lymph node metastasis. In hepatocellular carcinomas, high TP73 expression levels were revealed as an independent prognostic marker of poor survival (49). TP73 overexpression is more associated with CRC patients with tumor size larger than 5 cm in our study. BRCA1 had been reported to be involved in the radiotherapy resistance of breast cancer (50). In this study, BRCA1 overexpression is associated with CRC patients with deeper tumor invasion earlier cancer stage (T3+T4) with statistical significance.

ZAP-70 expression has been associated with disease progression in patients with chronic lymphocytic lymphoma (51). In the present study, ZAP-70 overexpression is associated with CRC patients with well and moderate differentiated histology. VAV1 was reported to act as a growth-stimulatory protein in primary pancreatic adenocarcinoma (52). VAV1 overexpression is noted associated with CRC patients with advanced cancer stage or with lymph node metastasis in this study.

Our results have medical implications. The genes particularly identified in this study will enable the genetic prediction of individualized sensitivity to RT. In addition, the identification of genes involved in regulating radiation response will enable the development of radiosensitizers that increase the sensitivity of tumor(s) to radiation. We suggest that pretherapeutic gene expression profiling may assist in response prediction of CRC to RT. The implementation of gene expression profiles for treatment stratification and clinical management of cancer patients requires validation in large independent studies.

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