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Effect of Ionizing Irradiation on Human Esophageal Cancer Cell Lines by cDNA Microarray Gene Expression Analysis

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Abstract

To provide new insights into the molecular mechanisms underlying the effect of irradiation on esophageal squamous cell carcinomas (ESCCs), we used a cDNA microarray screening of more than 4,000 genes with known functions to identify genes involved in the early response to ionizing irradiation. Two human ESCC cell lines, one each of well (TE-1) and poorly (TE-2) differentiated phenotypes were screened. Subconfluent cells of each phenotype were treated with single doses of 2.0 Gy or 8.0 Gy irradiations. After a 15 min incubation time-point, the cells were collected and analyzed. Compared with non-irradiated cells, many genes revealed at least 2-fold upregulation or downregulation at both doses in well or poorly differentiated ESCC cells. The common upregulated genes in well and poorly differentiated cell types at both irradiation doses included SCYA5, CYP51, SMARCD2, COX6C, MAPK8, FOS, UBE2M, RPL6, PDGFRL, TRAF2, TNFAIP6, ITGB4, GSTM3, and SP3 and common downregulated genes involved NFIL3, SMARCA2, CAPZA1, MetAP2, CITED2, DAP3, MGAT2, ATRX, CIAO1, and STAT6. Several of these genes were novel and not previously known to be associated with irradiation. Functional annotations of the modulated genes suggested that at the molecular level, irradiation appears to induce a regularizing balance in ESCC cell function. The genes modulated in the early response to irradiation may be useful in our understanding of the molecular basis of radiotherapy and in developing strategies to augment its effect or establish novel less hazardous alternative adjuvant therapies.
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Key words: esophageal squamous cell carcinoma (ESCC), cell line, radiation, gene expression, cDNA microarray

Introduction

Esophageal squamous cell carcinomas (ESCCs) are mostly associated with poor prognosis and the majority of patients die within 1 year of diagnosis[1]. Preoperative radiotherapy is an acceptable modality in the treatment of ESCCs. This mode of therapy is thought to improve resection rates, increase survival times, and decrease lymph metastases[2]. Furthermore, several studies have demonstrated that the inclusion of chemotherapy in this treatment

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modality improves a patient's outcome[3-5]. Hence chemoradiotherapy is now considered standard therapy in the non-surgical management of locally advanced ESCCs. This combination therapy has significantly improved median survival and reduced late relapses in patients with ESCCs[3]. In a randomized trial in patients with ESCCs, preoperative chemotherapy and concurrent radiotherapy significantly improved survival compared with surgery alone[4]. In another randomized trial in which the preoperative chemoradiotherapy was applied, a significant survival benefit for combined therapy was observed[5].

In addition to the preoperative radiotherapy, postoperative prophylactic radiotherapy has also been used in the treatment of ESCCs, but with contradictory results. In a multicenter controlled trial, postoperative radiotherapy did not increase survival after curative resection of ESCCs[6]. In contrast, a recent randomized prospective study on 495 ESCC patients showed that postoperative radiotherapy improved 5-year survival rate in patients with positive lymph node metastases and in patients with stage III disease compared with similar patients who did not receive radiotherapy[2]. These results signify the need for defining the molecular mechanism(s) of cellular response to ionizing irradiation that may provide clues for the development of strategies to augment its effect or establish novel less hazardous alternative adjuvant therapies.

In this study, we sought to obtain the gene expression profile of ESCC cells early after irradiation. Early alterations in gene expression levels appear more likely to involve genes participating in signaling pathways that determine the cell destiny. We cultured human ESCC cell lines to reach subconfluence and treated them with 2.0 Gy (a conventional daily dose during fractionated radiotherapy) or 8.0 Gy (usually applied for total-body irradiation) irradiation. These conditions approach the situation in clinical practice. After a 15 min incubation time, the cells were collected and analyzed using a cDNA microarray screening against more than 4,000 known genes. The subset of modulated genes was classified by clustering and biological functions were assigned to the genes which were then correlated with expression patterns. Functional annotations of the modulated genes could provide clues as to the effect of irradiation as well as suggesting putative genes as molecular targets for developing strategies to augment the irradiation effect or establish novel less hazardous alternative adjuvant therapies for ESCCs. These approaches may provide new insights into the molecular mechanisms involved in the irradiation response.

Materials and Methods

Cell Lines and Culture

Two human ESCC cell lines (obtained from Cancer Cell Repository, Institute of Development, Aging and Cancer, Tohoku University, Sendai, Japan), one each of well (TE-1) and poorly (TE-2) differentiated phenotypes were studied. The cell lines had been established from the resected primary esophageal tumors of male patients[7]. Cells were cultured in RPMI-1640 supplemented with 5% FBS, streptomycin (100 μg/ml), and penicillin (100 units/ml) at 37℃ in a humidified 5% CO_2 incubator.

Irradiation and Isolation of RNA

Subconfluent monolayer from each cell line was treated with 2.0 Gy or 8.0 Gy irradiation at 200 kV and 10 mA with 0.5 mm Cu and 1.0 mm Al filters. Non-irradiated paired preparations served as controls. After 15 min incubation time, the cells were collected for analyses. Total cellular RNA was prepared using the RNeasy Mini Kit (QIAGEN, Hilden, Germany). The RNA was treated with DNase and precipitated using 95% ethanol prior to cDNA synthesis. Isolated RNA was electrophoresed through 1.0% agarose-formaldehyde gels to verify the quality of the RNA, and RNA concentrations were determined by UV spectrophotometry.

cDNA Microarrays

The GeneFilters cDNA microarrays (Named Genes; GF211) were purchased from Research Genetics (Huntsville, AL). Each filter is a 5×7-cm

charged nylon membrane on which more than 4,000 sequence-verified human genes, all of which have known functions, are printed as individual immobilized spots.

Hybridization and Analysis of Microarrays

cDNA microarray filters were hybridized according to a protocol developed by the manufacturer (Research Genetics; http://www.resgen.com). One μg of total RNA was utilized as a template for a reverse transcriptase reaction (Superscript II, Invitrogen, Carlsbad, CA) to create [^{33}P] dCTP labeled cDNA probes. Reactions were purified by chromatography-columns (Bio-Spin 6, Bio-Rad Labs., Hercules, CA). The microarray filters were pre-washed in boiling 0.5% SDS for 5 min, placed individually in hybridization roller bottles with 5 m*l* MicroHyb solution, pre-hybridized with 5 μg denatured poly-dA and Cot-1 DNA (Research Genetics) for 2 h at 42℃, and then hybridized overnight with individual [^{33}P] labeled cDNA probes. The filters were washed for 20 min in hybridization bottles at 50℃ each with 2×SCC 3 times, 1% SDS 2 times, and 0.5×SCC/1% SDS 1 time. Moist filters were wrapped individually with plastic wrap, carefully oriented and exposed to phosphor-storage screens (Imaging Screen K, BioRad) in photographic cassettes for 16 h. Exposed screens were imaged using Molecular Imager FX (Bio-Rad) and tiff files were imported into Pathways 4.0 software (Research Genetics) for image alignment, translation of the raw hybridization intensities, and comparative analyses. Experiments were conducted independently two times. Genes that, after irradiation, showed at least 2-fold differences in their expression levels in at least one experimenal point in both duplicates were selected for cluster analysis. Biological functions of the genes were assigned by using the GENECARDS database [http://bioinformatics.weizmann.ac.il/cards/].

Semiquantitative RT-PCR Analysis

Aliquots of total cellular RNA (1.0 μg) were subjected to first-strand cDNA synthesis using Superscript II reverse transcriptase (Invitrogen), and the cDNA was diluted five times with water. One μ*l* of the diluted cDNA was used for each PCR reaction. The PCR primer set used for each gene; SCYA 5 was: forward, 5'-CGCTGTCATCC TCATTGCTA-3' and reverse, 5'-GCTGTCTCGAA CTCCTGACC-3'; the product size, 498 bp; SMARCD 2 was: forward, 5'-CTCGTCATTG AGCTGGACAA-3' and reverse, 5'-TCTCTGGGTC TTCAGCTGGT-3'; the product size, 597 bp; COX6C was: forward, 5'-GTCAGGAAGGACGTT GGTGT-3' and reverse, 5'-ACCAGCCTTCCTCA TCTCCT-3'; the product size, 262 bp; CAPZA 1 was: forward, 5'-TCGGATGAGGAGAAGG TACG-3' and reverse, 5'-ATCTTAAGCACGCCA ACCAC-3'; the product size, 557 bp; SMARCA 2 was: forward, 5'-AGACGGCTCTCAACTCCA AA-3' and reverse, 5'-AGGTCACTCATCTGGCT GCT-3'; the product size, 559 bp; MetAP 2 was: forward, 5'-ATG-GCGGGTGTGGAGGAGGT AGCGGCCT-3' and reverse, 5'-TTAATAGTCATC TCCTCTGCTGACAACT-3'; the product size, 1,440 bp; and DAP 3 was: forward, 5'-TGCCTGATGGT AAGGAAACC-3' and reverse, 5'-TCCCCAAAGAG CATTGATTC-3'; the product size, 519 bp. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control in RT-PCR reaction with primers: forward, 5'-TCCACCACC CTGTTGCTGTA-3' and reverse, 5'-ACCACAGT CCATGCCATCAC-3'; the product size, 450 bp. PCR was carried out for 24 cycles consisting of heat denaturation (94℃ for 1 min), annealing (55℃ for 1 min), and extension (72℃ for 3 min). The PCR products were electrophoresed on 2% agarose gels containing 0.1 mg/m*l* of ethidium bromide, and the gels were photographed under ultraviolet illumination. Semiquantitative analysis was done by densitometric scanning based on the standard curves constructed for each gene and GAPDH mRNAs. The analyses were performed using Molecular Imager FX and Quantity One Software Package (Bio-Rad).

Results

Gene Expression Analysis

In the well and poorly differentiated ESCC cell lines, many genes were modulated in early response

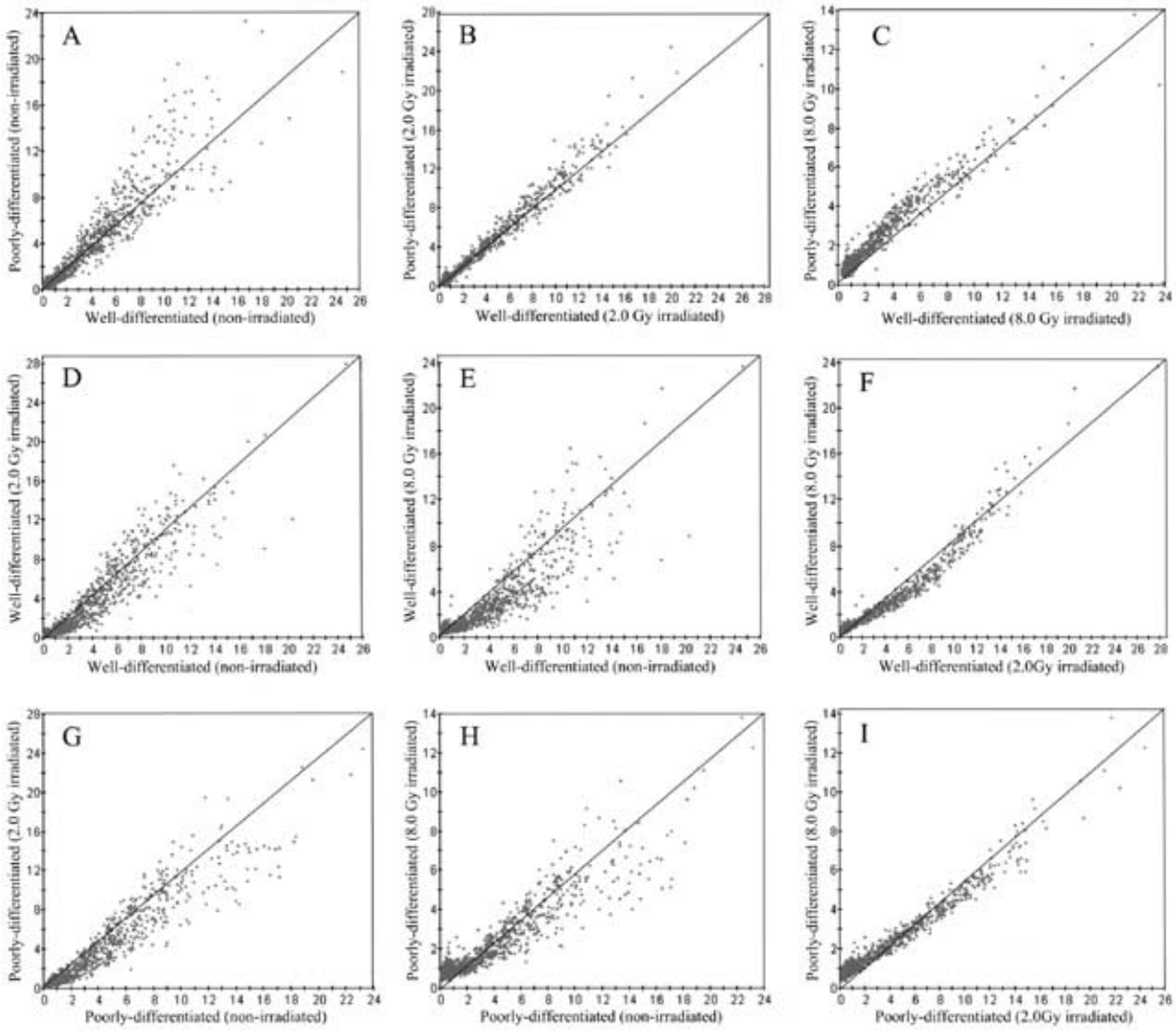

Fig. 1 Scatter plots of gene expression profiles for various pair-wise comparisons. Many genes were differentially expressed between well and poorly differentiated cell lines (A) that were mostly ameliorated after 2.0 Gy (B) or 8.0 Gy (C) irradiation. Well-differentiated cells at 2.0 Gy (D) or 8.0 Gy (E) irradiation showed modulation of many genes that when compared with each other (F) revealed amelioration of most of the modulated genes. Poorly-differentiated cells at 2.0 Gy (G) or 8.0 Gy (H) irradiation showed modulation of many genes that when compared with each other (I) revealed amelioration of most of the modulated genes. Numerical values indicate gene expression intensity.

to single dose of 2.0 Gy or 8.0 Gy of irradiation. Scatter plots of normalized intensity data from microarray experiments for various pair-wise comparisons are displayed in **Fig. 1**. Data points that lie along the diagonal of these scatter plots represent genes expressed at comparable levels in both sets, whereas those that lie off diagonal represent genes expressed at greater levels in the cell type identified by the nearer axis. By these demonstrations it was clear that many genes were differentially expressed between the well and poorly differentiated ESCC cell lines (**Fig. 1A**). These modulated genes were presumably accounted for by differences between the two phenotypes. Interestingly, the modulations were mostly ameliorated after 2.0 Gy (**Fig. 1B**) or 8.0 Gy (**Fig. 1C**) irradiations. The scatter plots of irradiated (2.0 Gy or 8.0 Gy) versus non-irradiated (control) cells for well differentiated (**Figs. 1D and 1E**) or poorly differentiated (**Figs. 1G and 1H**) ESCC cell lines also revealed many differentially expressed genes. These modulated genes were presumably accounted for by the early effect of irradiation.

Table 1 Upregulated genes at both doses of 2.0 Gy and 8.0 Gy irradiations in well or poorly differentiated ESCC cells

Gene *	Chromosome	Ratio	Function
SCYA5	17	3.02	Small inducible cytokine A5 (RANTES), chemoattractant for blood monocytes, T helper cells and eosinophils
CYP51	7	2.01	Involved in sterol biosynthesis
KRT18	12	2.63	Intermediate filament in cytoskeleton
SMARCD2	17	3.97	General transcriptional activator S cerevisiae SWI/SNF related protein
COX6C	8	2.19	Mitochondrial electron transport
CTNNA1	5	7.78	E-cadherin-mediated cell-cell adhesion associated protein
ANXA8	10	6.43	Selectively overexpressed in acute myelocytic leukemia
MAPK3	16	6.31	Participates in cell cycle progression
MAPK8	10	2.83	Involved in proapoptotic signal transduction
FOS	14	6.61	Transcription factor, major component of the activator protein-1 (AP-1) transcription factor complex, which includes members of the JUN family
B2M	15	2.21	Essential to expression of HLA antigen
GMFB	—	7.74	Intracellular regulator of signal transduction involving p38 MAP kinase
UBE2M	—	6.36	Ubiquitin-conjugating enzyme
CREB1	2	3.03	Required for the normal induction of the transcription factor AP1 and subsequent interleukin-2 production and cell cycle progression
RPL6	12	4.04	Ribosomal protein
RPL38	17	4.86	Ribosomal protein
CPNE1	20	2.01	Membrane trafficking
RAP2A	13	5.27	Ras-related protein with various functions
PDGFRL	8	5.42	Tumor suppressor
TRAF2	9	5.57	Subjects cells to TNF-induced apoptosis
TNFAIP6	2	5.83	TNF-induced protein involved in apoptosis
ETF1	5	5.22	Involves in the termination of translation in eukaryotes
ITGB4	17	5.84	Mediates cell-matrix or cell-cell adhesion, and transduced signals that regulate gene expression and cell growth
TXNL	18	5.46	Protect cells from oxidative stress
MGST2	4	5.38	Catalyze the conjugation of glutathione with a variety of xenobiotics and their reactive metabolites
GSTM3	1	5.43	Involves in the metabolism of a broad range of xenobiotics and carcinogens
SP3	2	6.85	Transcriptional regulator

* Bold script depicts common upregulated genes in both well and poorly differentiated cell types.

Scatter plot of comparison of gene expression between 2.0 Gy and 8.0 Gy irradiations in the well (**Fig. 1F**) or poorly (**Fig. 1I**) differentiated cell lines showed that the modulated genes were mostly ameliorated after irradiation. Compared with non-irradiated cells, many genes revealed at least 2-fold upregulation or downregulation at each dose of irradiation in the well or poorly differentiated ESCC cells. The modulated genes at both doses in the well or poorly differentiated ESCC cells are summarized in **Table 1** and **Table 2**. The common upregulated genes in well and poorly differentiated cell types at both irradiation doses included SCYA5, CYP51, SMARCD2, COX6C, MAPK8, FOS, UBE2M, RPL6, PDGFRL, TRAF2, TNFAIP6, ITGB4, GSTM3, and SP3 and common downregulated genes involved NFIL3, SMARCA2, CAPZA1, MetAP2, CITED2, DAP3, MGAT2, ATRX, CIAO1, and STAT6.

Semiquantitative RT-PCR

Of the altered genes, we confirmed the expression of a selected number of genes including 3 upregulated (SCYA5, SMARCD2 and COX6C) and 4 downregulated (CAPZA1, SMARCA2, MetAP2 and DAP3) genes by gene-specific semiquantitative RT-PCR. These genes were selected randomly from among the genes commonly modulated in both well and poorly differentiated cell types. RNA samples from the same stocks as used for the cDNA microarray hybridization were examined. In each

Table 2 Downregulated genes at both doses of 2.0 Gy and 8.0 Gy irradiations in well or poorly differentiated ESCC cells

Gene *	Chromosome	Ratio	Function
SCYA3	17	0.45	Early G0/G1 switch gene in cultured blood monocytes, mononuclear cells, mediator of virus-induced inflammation in vivo
NFIL3	9	0.34	Regulates a pivotal step in an antiapoptotic signaling pathway in B lymphocytes
SMARCA1	X	0.29	Global activator of transcription in yeast, unknown function in mammals
SMARCA2	9	0.44	Regulator of chromatin, transcriptional activator during mitosis
SCNN1A	12	0.48	Involves in a nonvoltage-gated sodium channel activity
CAPZA1	1	0.23	Bind in a Ca(2+)-independent manner to the fast growing ends of actin filaments and blocks the exchange of subunits
FKBP8	19	0.32	May inhibit apoptosis by anchoring BCL2 and BCLXL to mitochondrial membranes
RAB2L	6	0.42	Membrane trafficking
PTHR1	3	0.46	Binds to the sodium/hydrogen exchanger regulatory factors NHERF1 and NHERF2, regulate PTH signaling
IGFBP6	12	0.31	Autocrine growth inhibitory role
MetAP2	12	0.28	Binds to eukaryotic initiation factor-2 (eIF-2), promotes protein synthesis in the cell
FDPS	—	0.45	Involves in liver peroxisomal deficiency diseases
SILV	12	0.48	Melanocytes protein
CITED2	6	0.49	Involves in transcription activation
HFL1	1	0.50	Codes factor H-related proteins
LCP1	13	0.32	Actin-binding protein
DAP3	1	0.20	Mediator of interferon-gamma-induced cell death
MGAT2	14	0.32	Golgi enzyme catalyzing an essential step in the conversion of oligomannose to complex N-glycans
ATRX	X	0.29	Nucleotide excision repair and initiation of transcription
ANXA13	8	0.28	Calcium-dependent phospholipid-binding proteins
GPC5	13	0.30	A cell surface proteoglycan modulating signaling
CIAO1	2	0.28	Inhibits the transcriptional activation of WT1 involved in regulating cell cycle progression and apoptosis
EIF2B5	3	0.34	Translation initiation factor
STAT6	12	0.30	Involves in signal transduction and activation of transcription
THBD	20	0.45	Converts thrombin into a physiologic anticoagulant
CETN2	X	0.30	Encodes the calcium-binding protein, centrin

* Bold script depicts common downregulated genes in both well and poorly differentiated cell types.

case RT-PCR data confirmed the gene expression data obtained by microarray hybridization (**Fig. 2**).

Discussion

Early modulations in gene expression associated with ionizing irradiation were studied in ESCC cells in order to identify potential targets for investigations into the mechanisms behind the effect of irradiation. Many studies on the effect of irradiation on gene expression have been performed using irradiation doses that are highly toxic and are far greater than those used in clinical therapy. Hence, we selected 2.0 Gy, a conventional daily dose used during fractionated radiotherapy in clinic, and 8.0 Gy, a dose often applied for total-body irradiation in patients.

The doubling time and radiation sensitivity of the TE-1 and TE-2 cell lines had been previously reported[8]. The doubling time was 25.2 and 14.4; *D0* (dose required to reduce the fraction of surviving cells to 37% of its previous value in exponential region of the survival curves) was 1.40 and 1.35; *Dq* (quasi-threshold dose to measure the shoulder width of survival curves) was 3.70 and 1.95; *SF2* (difference in survival fraction at 2.0 Gy between cell lines) was 0.848 and 0.380 respectively for the TE-1 and TE-2 cells. The doubling time, *SF2* and *Dq* of the TE-1 cells were greater than in TE-2 cells. However, no significant correlation was found between the

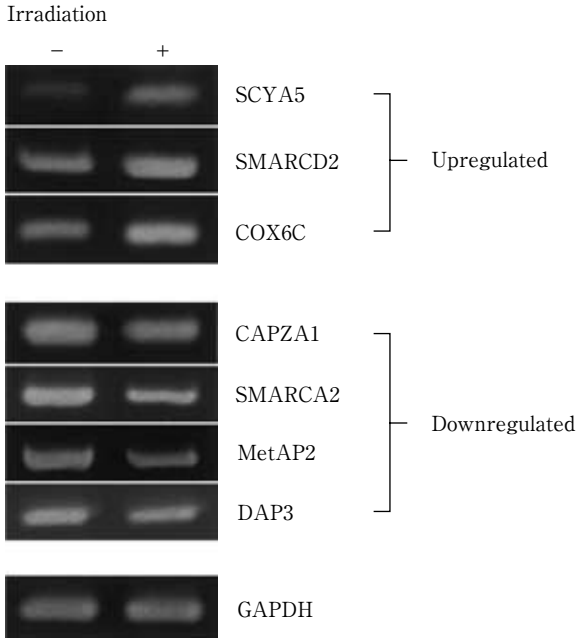

Fig. 2 Validation of gene expression data after irradiation by semi-quantitative RT-PCR. The differential expression levels of the selected genes were consistent with the results obtained from microarray analysis. GAPDH was used as an internal control.

doubling time and these values.

We first performed a pair-wise comparison between the well and poorly differentiated ESCC cells to identify the overall differences in gene expression profiles between the two phenotypes. Although many genes were differentially modulated as depicted in **Fig. 1A**, their further evaluation was beyond the scope of the present study. Then, we compared gene expression pattern of each cell line before and 15 min after irradiation at each dose to identify early modulated genes. By excluding the genes that were modulated due to the phenotypic effect from the data, we were able to assess genes that were more likely affected by irradiation itself. We further sought to extract from the data those modulated genes that were common among the comparison pairs at both doses of irradiation. The cellular response to irradiation is mediated via genes that control complex regulatory pathways. These pathways may involve induction of apoptosis, cell cycle retardation, and interference with DNA repair as desirable effects of irradiation or alternatively inhibition of apoptosis, cell cycle acceleration, and activation of DNA repair as resisting measures against irradiation effects. These alterations could either augment cell death or protect cells from the lethal effects of irradiation. Our results demonstrated upregulation of several genes that promote cell death or inhibit cell growth in parallel with downregulation of several genes that inhibit cell death or promote cell growth. These findings suggested that at the molecular level, irradiation appears to induce a regularizing balance in ESCC cell function.

Previous studies have identified several genes whose expression has been modulated by ionizing irradiation, including the oncogene and transcription factor c-jun[9], cell growth regulating cyclin B 1[10], basic fibroblast growth factor[11], p53[12], cytoskeletal and matrix genes[13], TNF-alpha[14] and interferon-gamma[15]. Our study has permitted the identification of a number of putative genes not previously known to be associated with irradiation response. Several of these novel genes, particularly the downregulated genes, deserve comment. Annexin A13 (ANXA13) is believed to be the original founder gene of the 12-member vertebrate annexin A family, and it has acquired an intestine-specific expression associated with a highly differentiated intracellular transport function[16]. CAPZA1 is a member of the F-actin capping protein alpha subunit family. This gene encodes the alpha subunit of the barbed-end actin binding protein that regulates growth of the actin filament by capping the barbed end of growing actin filaments[17]. MetAP2 is a 67-kD protein, termed p67, which binds to eukaryotic initiation factor-2 (eIF-2). Wu et al. (1993)[18] showed that p67 protects eIF-2-alpha from phosphorylation by eIF-2 kinases and thus promotes protein synthesis in the cell. They suggested that p67 may play a critical role in the regulation of protein synthesis. MGAT2 is a member of monoacylglycerol acyltransferase (MGAT) or Acyl CoA that catalyzes the synthesis of diacylglycerol, a precursor of triacylglycerol. In humans, the MGAT2 gene is highly expressed in the small intestine, stomach, colon, liver, kidney, and white adipose tissue[19]. DAP3 (death-associated protein 3) is a nucleotide-binding protein which is increased due to

interferon-gamma induced cell death[20]. Furthermore, it has been shown that ionizing irradiation reduces the interferon-gamma mRNA expression[15]. It has also been found that DAP3 binds directly to the death domain of TNF-related apoptosis-inducing ligand (TRAIL) receptors, and is required for TRAIL-induced apoptosis[21]. These observations may explain the underlying mechanism and relevance of DAP3 downregulation after irradiation in our study. In addition, from the upregulated genes, SCYA5 or RANTES is a small inducible cytokine involved in the inflammatory process and has been found to be elevated in radiation-induced pulmonary injury and subsequent fibrosis in mice[22]. COX6C is a cytochrome c oxidase (COX) subunit that functions in electron transfer. COX is the terminal enzyme of the mitochondrial respiratory chain. It catalyzes the electron transfer from reduced cytochrome c to oxygen, and is involved in the process of apoptosis. Further studies are needed to elucidate the putative roles of these newly identified genes in signaling pathways targeted by ionizing irradiation.

An interesting finding in our study was the observation that the global pattern of gene expression after irradiation as judged by scatter plots was highly informative. As shown in **Fig. 1** scatter plots of gene expression patterns for pair-wise comparisons could provide information on the effect of each treatment. Many genes were differentially expressed between well and poorly differentiated cell lines before irradiation, which were accounted for by the differences between the two phenotypes and perhaps attributable to the worsening of the phenoptype (**Fig. 1A**). After irradiation with 2.0 Gy or 8.0 Gy dose, these differentially expressed genes were mostly ameliorated (**Figs. 1B and 1C**). This finding further indicated that at the molecular level, irradiation may induce a regularizing balance in ESCC cell function. Moreover, in well and poorly differentiated cells, many genes were modulated in response to 2.0 Gy (**Figs. 1D and 1G**) or 8.0 Gy (**Figs. 1E and 1H**) irradiation. These genes appeared to be most common between the two treatment doses, as judged from the scatter plots of comparison between 2.0 Gy and 8.0 Gy irradiation in the two cell types (**Figs. 1F and 1I**). This observation suggested that the gene expression responses may be independent from irradiation dose. Previous studies have also noted similar trends using different cell systems[23,24].

In summary, we compared the gene expression profiles of ESCC cells before and after irradiation using cDNA microarray screening, and identified several genes that were modulated in early response to irradiation. These genes may be useful in our understanding of the molecular basis of radiotherapy and in developing strategies to augment its efficacy or establish novel less hazardous alternative adjuvant therapies. They may also provide novel targets for basic research or potential biomarkers that might be of clinical relevance. We are currently examining the expression levels of several of these putative genes in human ESCC tissues in relation to their radiosensitivity or radioresistance nature.

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