S100A4 mRNA expression level is a predictor of radioresistance of pancreatic cancer cells

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Abstract. Improving poor outcomes in patients with pancreatic cancer requires a greater understanding of the biological mechanisms contributing to radioresistance. We, therefore, sought to identify genes involved in the radioresistance of pancreatic cancer cells. Two pancreatic cancer cell lines, CFPAC-1 and Capan-1, were repeatedly exposed to radiation, establishing two radioresistant cell lines. Gene expression profiling using cDNA microarrays was performed to identify genes responsible for radioresistance. The levels of expression of mRNAs encoded by selected genes and their correlation with radiation dose resulting in 50% survival rate were analyzed in pancreatic cancer cell lines. The radiation dose resulting in a 50% survival rate was significantly higher in irradiated (IR) compared to parental CFPAC-1 cells (8.31±0.85 Gy vs. 2.14±0.04 Gy, P<0.0001), but was lower in IR compared with parental Capan-1 cells (2.66±0.24 Gy vs. 2.25±0.03Gy, P=0.04). cDNA microarray analysis identified 4 genes, including S100 calcium binding protein A4 (S100A4), overexpressed and 23 genes underexpressed in the IR compared with the parental cell lines. The levels of S100A4 mRNA expression were correlated with radiation dose resulting in a 50% survival rate (Pearson's test, R^2=0.81, P=0.0025). S100A4 mRNA expression may predict radioresistance of pancreatic cancer cells and may play an important role in the poor response of pancreatic cancer cells to radiation therapy.

Introduction

Pancreatic cancer is the fourth leading cause of cancer-related mortality in Western countries and has the lowest survival rate of any solid tumor (1). Although patients with pancreatic cancer are treated with comprehensive therapy, including surgery, chemotherapy and radiotherapy, their 5-year survival rate is <5% (2). Approximately 25-30% of these patients are initially diagnosed with locally advanced pancreatic cancer (3,4). Although radiotherapy combined with chemotherapy is the recommended treatment strategy, the contribution of radiotherapy is unclear (3,4). To improve the outcome of patients with pancreatic cancer, a greater understanding of the biological mechanisms that contribute to tumor refractoriness to radiotherapy is required. Moreover, identification of markers of radioresistance may contribute to the identification of agents to treat this fatal disease.

The cDNA microarray technique is a powerful tool for analyzing comprehensive gene expression in cells (5). To identify genes that contribute to resistance to radiation therapy, cDNA microarray analysis has compared radioresistant and radiosensitive cancer cells in various types of tumor, including uterine cervical, head and neck, colorectal, breast, esophageal, lung, hepatocellular and pancreatic cancer, as well as in malignant melanoma (6). Several genes identified by cDNA microarray analysis encode proteins involved in apoptosis, DNA repair, signal transduction, cell cycle and cell adhesion, as well as genes encoding growth factors and growth factor receptors (6). Although these genes may contribute to radioresistance, more information is required to evaluate the mechanism underlying resistance to radiotherapy in these tumor types.

We therefore exposed pancreatic cancer cell lines to radiation and compared comprehensive gene expression of irradiated (IR) and parental cells using cDNA microarray technique. We selected promising genes and evaluated their contribution to the radioresistance of pancreatic cancer cell lines.

Materials and methods

Cell lines. Three human pancreatic cancer cell lines, SUIT-2, PANC-1 and MIA PaCa-2, were obtained from the Japanese Cancer Resource Bank (Tokyo, Japan) and three other human pancreatic cancer cell lines, Capan-1, Capan-2 and CFPAC-1, were obtained from the American Type Culture Collection (Manassas, VA, USA); all 6 cell lines bear mutant p53 (7-9). The cells were maintained as previously described (10).

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Irradiation of pancreatic cancer cell lines. Two pancreatic cancer cell lines, Capan-1 and CFPAC-1, were repeatedly exposed to 2 Gy X-ray irradiation for a total dose of 10 Gy, followed by irradiation with 5 Gy to a total dose of 60 Gy, with 14-day intervals between treatments. IR cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS.

Evaluation of cell rediosensitivity. Radiosensitivity was assessed by colony formation assays. All pancreatic cell lines were plated at $1x10^4$ cells/well in 6-well plates (Thermo Scientific, Rockford, IL, USA) for 24 h, followed by exchange of conditioned medium. The cells were irradiated at 0, 1, 2, 3, 5, 7, 8 and 10 Gy and incubated for 7 days without exchange of medium. The plates were fixed in 70% ethanol and stained with 0.1% crystal violet and colonies were counted using a ChemiDoc XRS System (Bio-Rad Laboratories). Survival rates were determined by comparing the number of colonies at each radiation dose with that in unirradiated (0 Gy) cells. All survival curves represent the combined results of three independent experiments.

Microarray analysis. Microarray analyses were performed using IR-Capan-1 and IR-CFPAC-1 cells and their respective parental cells. The qualities of the RNA samples were evaluated using a 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany) as described (11). Analyses were performed using a HumanWG-6 Expression BeadChip and the results were analyzed using BeadStudio software version 3.2.3 (both from Illumina, San Diego, CA, USA).

Real-time qRT-PCR. Total RNA was extracted from pellets of cultured cells using a High Pure RNA Kit (Roche Diagnostics, Mannheim, Germany) and treated with DNase I (Roche Diagnostics), according to the manufacturer's instructions. Specific primers for S100 calcium binding protein A4 (*S100A4*) (forward, 5'-atcgccatgatgtgtaccga-3'; reverse, 5'-cccaaccacatcagagg agt-3') and *β-actin* (forward, 5'-tgagcgc-ggctacagctt-3'; reverse, 5'-tccttaatgtcacgcacgattt-3') RNAs were designed using primer 3, with BLAST searches performed to ensure the specificity of each primer. The extracts were analyzed by qRT-PCR using a QuantiTect SYBR-Green RT-PCR Kit (Qiagen, Tokyo, Japan) and a Chrom4 Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA). Each reaction mixture was initially incubated at 50˚C for 30 min to allow reverse transcription, in which first-strand cDNA was synthesized by priming the total RNA with the same gene-specific primer (reverse). PCR was initiated by incubation at 95˚C for 15 min to activate the polymerase, followed by 40 cycles of 94˚C for 15 sec, 58˚C for 30 sec and 72˚C for 30 sec. Each of these primer sets produced a single prominent band of the expected size after electrophoresis. Each sample was analyzed twice and any samples showing >10% deviation in qRT-PCR results were tested a third time. The level of mRNA expression in each sample was calculated by reference to a standard curve generated using total RNA from the SUIT-2 human pancreatic cancer cell line. The expression of *S100A4* mRNA was normalized relative to the expression of *β-actin* mRNA in the same sample.

Western blotting. Cell proteins were extracted by PRO-PREP (iNtRON Biotechnology, Seongnam, Korea) according to the manufacturer's instructions. Cell lysates containing 15 μg protein were fractionated on 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels and transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). Each membrane was incubated overnight at 4˚C with anti-S100A4 (1:500; DAKO, Glostrup, Denmark) or anti-β-actin (05-829, 1:1,000; Millipore) and subsequently with secondary antibodies conjugated to horseradish peroxidase (Santa Cruz Biotechnology, Inc.). Immunoblots were detected by enhanced chemiluminescence with a Chemi Doc XRS System.

Cell proliferation assay. Cell proliferation was evaluated by measuring the fluorescence intensity of propidium iodide (PI), as previously described (12). Briefly, cells were seeded at $2x10^4$ cells/well in 24-well tissue culture plates (Becton Dickinson Labware, Bedford, MA, USA) and cultured in DMEM containing 10% FBS for 24 h. Following confirmation of cellular adhesion to the plates, the medium was replaced and 30 μM PI and 600 μM digitonin were added to each well to label the nuclei with PI. The fluorescence intensity, corresponding to the total cell number, was measured using an Infinite® 200 PRO multiwell plate reader (TECAN, Mannedorf, Switzerland) with excitation and emission wavelengths of 530 and 645 nm, respectively. A separate well containing the same medium was used to provide a baseline PI signal as a control. The difference in intensity between each sample well and the control well was calculated. Cell proliferation was defined relative to the number of cells counted immediately after exchanging the medium. All experiments were performed in triplicate wells and were repeated at least three times.

Statistical analysis. All statistical analyses were performed using JMP 8.0.1 software (SAS Institute, Inc., Cary, NC, USA). Pearson's test was measured to evaluate the relationships between S100A4 mRNA expression and radiation dose associated with 50% cell survival, S100A4 mRNA expression and relative proliferation of pancreatic cancer cells and relative proliferation of pancreatic cancer cells and radiation dose associate with 50% cell survival. All values are expressed as means ± standard deviation. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Evaluation of radiosensitivity of IR-CFPAC-1 and Capan-1 cells. Two pancreatic cancer cell lines, CFPAC-1 and Capan-1, were exposed to fractionated irradiation until the total irradiation dose was 60 Gy. Radiosensitivity was evaluated by the colony formation assay. IR-CFPAC-1 cells showed significantly greater radioresistance compared to parental cells. The radiation dose resulting in a 50% survival rate was significantly higher in IR compared to parental CFPAC-1 cells (8.31±0.85 Gy vs. 2.14±0.04 Gy, $P<0.0001$; Fig. 1A and B), but the difference was less pronounced in IR compared to parental Capan-1 cells (2.66±0.24 Gy vs. 2.25±0.03 Gy, $P=0.04$).

Table I. Average ratios of expression of upregulated genes in radioresistant compared with parental cells (fold-change >2).

Rank	Gene name	Fold-change		Function	Sequence code
		CFPAC-1	Capan-1		
1	S100 calcium binding protein A4 (S100A4)	138.3	8.5	Regulation of a number of cellular processes such as cell cycle progression and differentiation	NM_019554.2
2	Transmembrane protein 158 (TMEM158)	18.1	3.2	Upregulated in response to activation of the Ras pathway, but not under other conditions that induce senescence	NM_015444.2
3	Caveolin 2 (CAV2)	16.3	2.4	Signal transduction, lipid metabolism, cellular growth control and apoptosis	NM_001233.3
4	Phosphoprotein enriched in astrocytes 15 (PEA15)	5.5	2.1	Regulation of apoptosis	NM_003768.2

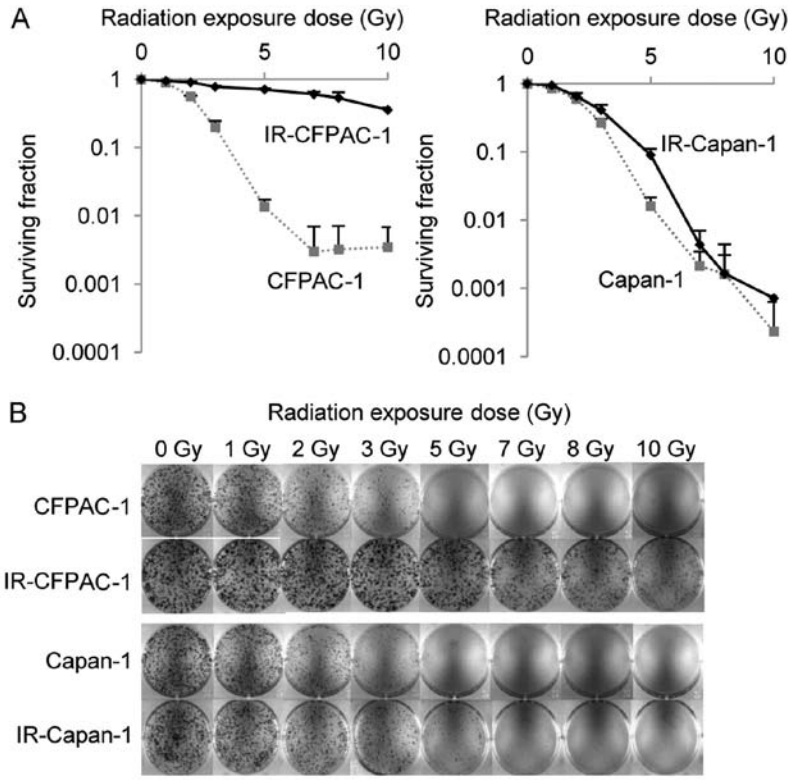

Figure 1. Radiosensitivity of parent and irradiated (IR) CFPAC-1 and Capan-1 cells. (A) Radiosensitivity was evaluated by the colony formation assay. IR-CFPAC-1 showed significantly greater radioresistance than parental cells, with radiation doses associated with 50% survival rates of 8.31±0.85 and 2.14±0.04 Gy, respectively (P<0.0001). Irradiation had a reduced effect on Capan-1 cells, with radiation doses associated with 50% survival rates of IR-Capan-1 and Capan-1 cells of 2.66±0.24 and 2.25±0.03 Gy, respectively (P=0.0426). (B) Representative images of colonies formed by each cell line following irradiation.

mRNA expression profiling of IR and parental pancreatic cancer cell lines. We performed global microarray expression analysis of 48,803 genes to compare two IR pancreatic cancer cell lines (IR-CFPAC-1 and IR-Capan-1) with those of their respective parental cell lines (CFPAC-1 and Capan-1). A difference was defined as significant if the ratio of expression between IR and parental cells was >2- or <0.33-fold. We found that 4 genes were upregulated (Table I) and 23 were downregulated (Table II) in IR compared with parental cells. One of the overexpressed genes was S100A4, the increased expression of which has been associated with radiation exposure in lung cancer cell lines (13). Of the 23 downregulated genes, none of which has been reported to be associated with radioresistance, 7 were potentially involved in apoptosis, cell attachment and inhibition of cell proliferation and tumor suppression.

S100A4 expression in cultured pancreatic cancer cell lines. We investigated the levels of expression of S100A4 mRNA in cultures of 6 unirradiated and 2 IR pancreatic cancer cell lines. S100A4 mRNA expression was higher in both IR cell lines

Table II. Identification of the 23 genes downregulated in radioresistant CFPAC-1 and Capan-1 cells compared with their respective parental cells (fold-change <0.33).

Rank	Gene name	Fold-change		Function	Sequence code
		CFPAC-1	Capan-1		
1	Inhibitor of DNA binding 3 (ID3)	0.02	0.27	Negative regulation of transcription factor activity	NM_002167.2
2	Odontogenic, ameloblast associated (ODAM)	0.03	0.15	Odontogenesis	NM_017855.3
3	Rho-related BTB domain containing 3 (RHOBTB3)	0.03	0.19	Small GTPase-mediated signal transduction and the organization of the actin filament system	NM_014899.3
4	Eukaryotic translation elongation factor 1 α2 (EEF1A2)	0.04	0.07	Protein biosynthesis, translational elongation	NM_001958.2
5	Naked cuticle homolog 2 (*Drosophila*) (NKD2)	0.07	0.30	Negative regulator of the Wnt in the mouse	NM_033120.2
6	Quinolinate phosphoribosyl-transferase (QPRT)	0.08	0.04	Generation of precursor metabolites and energy, NAD biosynthesis, synaptic transmission	NM_014298.3
7	Imprinted maternally expressed transcript (H19)	0.08	0.05	Non-coding RNA and functions as a tumor suppressor	NR_002196.1
8	Homo sapiens laminin, α2 (LAMA2)	0.10	0.09	Regulation of embryonic development, muscle development, cell migration and adhesion	NM_001079823.1
9	Vasorin (VASN)	0.13	0.28	Vasorin was reported to contribute to neointimal formation after vascular injury	NM_138440.2
10	AT rich interactive domain 5B (MRF1-like) (ARID5B)	0.15	0.32	Negative regulation of transcription	NM_032199.1
11	G protein-coupled receptor 56 (GPR56)	0.15	0.26	Cell adhesion, cell-cell signaling, signal transduction	NM_005682.4
12	Vaccinia related kinase 2 (VRK2)	0.17	0.33	Protein amino acid phosphorylation	NM_006296.3
13	Similar to CG14853-PB (LOC285141)	0.17	0.26		XM_939141.1
14	Growth arrest-specific 6 (GAS6)	0.17	0.32	Cell proliferation	NM_000820.1
15	T-box 3 (ulnar mammary syndrome) (TBX3)	0.17	0.29	Morphogenesis, skeletal development, regulation of transcription from RNA polymerase II promoter	NM_016569.3
16	Collagen, type VII, α1 (COL7A1)	0.21	0.20	Cell adhesion, phosphate transport, epidermis development	NM_000094.2
17	Cystic fibrosis transmembrane conductance regulator (CFTR)	0.21	0.20	Ion transport, respiratory gaseous exchange	NM_000492.3
18	Synaptotagmin-like 2 (SYTL2)	0.22	0.15	Vesicle-mediated transport, intracellular protein transport	NM_032943.2
19	POM and ZP3 fusion (POMZP3)	0.25	0.18		NM_152992.2
20	Prominin 1 (PROM1)	0.28	0.06	Visual perception	NM_006017.1
21	Suppressor of cytokine signaling 2 (SOCS2)	0.32	0.19	JAK-STAT cascade, negative regulation of signal transduction, anti-apoptosis, regulation of cell growth	NM_003877.3
22	Shroom family member 4 (SHROOM4)	0.32	0.16	To play a role in cytoskeletal architecture	NM_020717.2
23	Angiopoietin 1 (ANGPT1)	0.33	0.16	Angiogenesis, cell differentiation, signal transduction	NM_001146.3

Table III. Radiation dose associated with 50% survival rates of 6 pancreatic cancer cell lines and IR cell lines.

Cell line	Radiation dose (Gy)
IR-CFPAC-1	8.31±0.85
SUIT-2	7.80±1.07
PANC-1	5.94±0.66
MIA PaCa-2	4.90±0.64
IR-Capan-1	2.66±0.24
Capan-1	2.25±0.03
CFPAC-1	2.14±0.04
Capan-2	2.04±0.32

IR, irradiated.

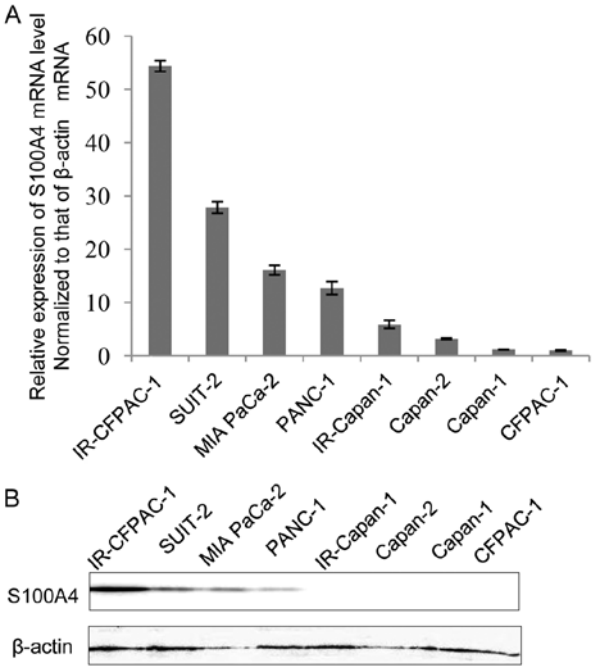

Figure 2. S100A4 expression in 6 pancreatic cancer cell lines and 2 irradiated (IR) cell lines. (A) S100A4 mRNA expression was analyzed by qRT-PCR and normalized relative to β-actin mRNA. S100A4 mRNA expression levels were 54.4- and 5.0-fold higher in IR-CFPAC-1 and IR-Capan-1 cells, respectively, compared to their respective parental cell lines. (B) S100A4 protein level was significantly higher in IR-CFPAC-1 compared to CFPAC-1 cells, whereas S100A4 protein was not detected in IR-Capan-1 or Capan-1 cells.

compared to the unirradiated cells, a finding consistent with our microarray data (Fig. 2A). Compared with their respective parental cell lines, the levels of expression of S100A4 mRNA were 54-fold higher in IR-CFPAC-1 compared to CFPAC-1 cells and 5.0-fold higher in IR-Capan-1 compared to Capan-1 cells, with both differences being statistically significant. The level of S100A4 protein was significantly higher in IR-CFPAC-1 compared to IR-Capan-1 cells, the latter of which, as well as parental Capan-1 cells, expressed no S100A4 protein (Fig. 2B). S100A4 protein and mRNA expression were closely correlated in these cell lines. These findings suggest that the difference in radiosensitivity between IR-CFPAC-1 and IR-Capan-1 cells may be due to differences in S100A4 expression.

S100A4 mRNA expression is significantly correlated with radiation dose associated with 50% survival. To analyze the radiosensitivity of pancreatic cell lines, we calculated the radiation dose associated with 50% survival rates (Fig. 3A and B, Table III). We found that S100A4 mRNA expression was significantly correlated with this radiation dose (Pearson's test, R^2=0.81, P=0.0025) (Fig. 4A) but not with cell proliferation (Pearson's test, R^2=0.04, P=0.62) (Fig. 4B). In addition, radiation dose associated with 50% cell survival was not correlated with cell proliferation (Pearson's test, R^2=0.10, P=0.45) (Fig. 4C). These findings indicate that S100A4 mRNA expression was closely associated with the radioresistance of pancreatic cancer cell lines.

Discussion

Radiotherapy is among the key treatment strategies for patients with various types of cancer. Some patients benefit from radiotherapy, whereas others do not. In particular, the survival benefits of radiotherapy in patients with pancreatic cancer remain unclear (3,4). The molecular mechanisms associated with tumor response to radiotherapy have been assessed in several types of tumor by cDNA microarray analysis, comparing radiosensitive and radioresistant cancer. These include esophageal cancer (14), head and neck cancer (15), cervical cancer (16-18), breast cancer (19), pancreatic cancer (6), glioblastoma (20), hepatocellular carcinoma (21), malignant melanoma (22) and lung cancer (23,24). Most of the genes identified in these cDNA arrays were associated with DNA-repair, apoptosis, growth factors, signal transduction, cell cycle and cell adhesion. Similarly, our cDNA microarray analysis also identified several genes involved in these signaling pathways. However, none of these genes has been identified in previous cDNA microarrays and some tumor types have few genes in common that are up- or downregulated. Thus, the mechanism of radioresistance in cancer cells is very complicated.

In analyzing pancreatic cancer cells, we found that S100A4 mRNA expression was markedly upregulated following irradiation, with the degree of upregulation differing significantly in strongly radioresistant IR-CFPAC-1 cells and weakly radioresistant IR-Capan-1 cells. Irradiation of lung cancer cell lines has been reported to increase the expression of S100A4 and S100A6 proteins (13). To our knowledge, our study is the first to show that the level of expression of S100A4 mRNA is directly correlated with radioresistance of pancreatic cancer cells.

S100A4 is a member of the S100 family of calcium binding proteins, which are characterized by two distinct EF-hand structural motifs (25,26). S100A4 is overexpressed in a number of solid tumors, including breast, esophageal, gastric, colorectal and pancreatic cancer and is associated with poor prognosis in patients with these types of cancer (27,28). S100A4 has been reported to promote cell motility and invasion in cancer and to be associated with tumor metastasis (29,30). Although we observed an association between S100A4 expression and radioresistance of pancreatic cancer cell lines, the mechanism by which S100A4 induces radioresistance remains unclear.

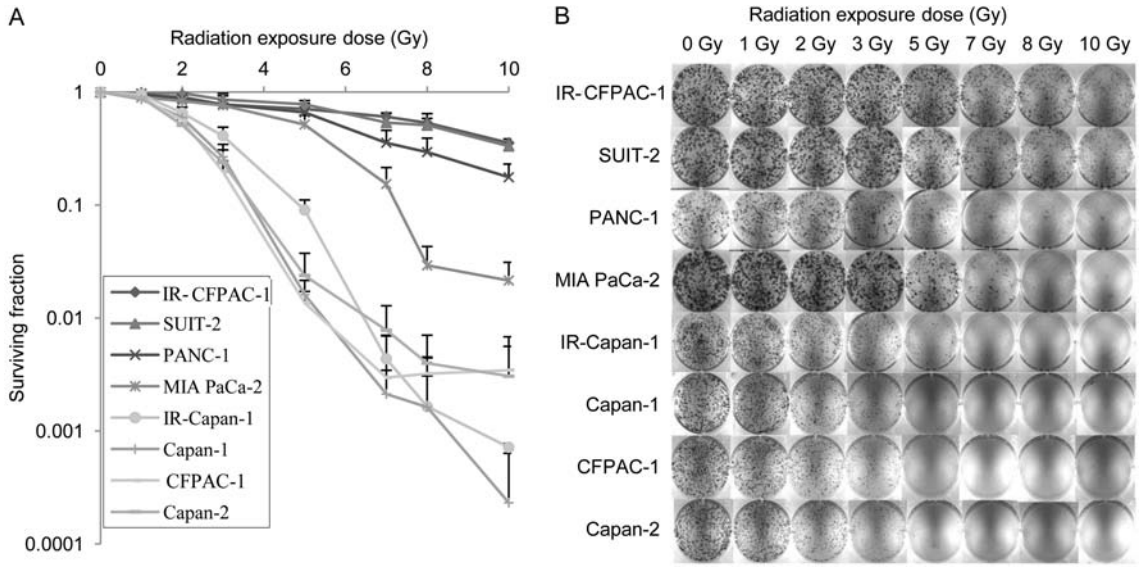

Figure 3. Radiosensitivity of pancreatic cancer cell lines. (A) Radiation cell survival curves of 6 pancreatic cancer cell lines and 2 irradiated (IR) cell lines. (B) Representative images of colonies formed by these cell lines following irradiation.

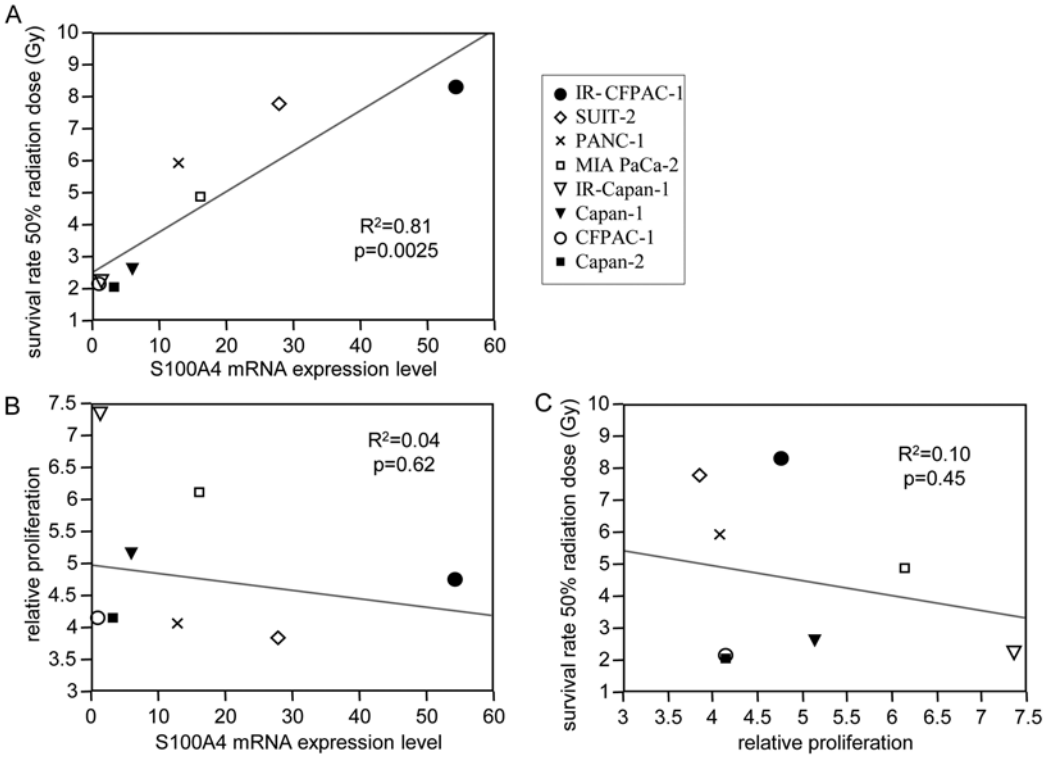

Figure 4. Correlation among S100A4 mRNA expression level, radiation dose associated with 50% survival rate and relative proliferation in 6 pancreatic cancer cell lines and 2 irradiated (IR) cell lines. (A) Significant correlation between S100A4 mRNA expression level and 50% radiation dose (Pearson's test, R^2=0.81, P=0.0025). (B) No significant correlation between S100A4 mRNA expression level and relative proliferation (Pearson's test, R^2=0.04, P=0.64). (C) No significant correlation between relative proliferation and 50% radiation dose (Pearson's test, R^2=0.10, P=0.45).

Wild-type p53 has been shown to play a crucial role in cellular responses to radiation-induced DNA damage through cell cycle arrest, apoptosis and DNA repair (31,32). By contrast, mutant p53 has been shown to be involved in resistance to radiotherapy (33-36) and to have oncogenic properties by inducing the expression of sets of genes that activate cell proliferation, cell survival and angiogenesis (35). S100A4 has been reported to interact with wild-type p53 through the C-terminal domain of the latter and may regulate p53 function, inducing cell apoptosis (37). Moreover, S100A4 may interact with mutant p53 (37). Activation of c-myc gene expression by mutant p53 has been reported to require the C-terminal domain of the latter (38). The pancreatic cancer cells utilized in this study contain mutant p53 (7-9). These findings suggest that upregulation of S100A4 may increase its opportunities to interact with mutant p53, enhancing resistance to radiation.

The NF-κB pathway has been reported to play an important role in the development of resistance to radiotherapy (39-41). Inhibition of the NF-κB pathway decreased resistance to radiotherapy. Moreover, release of S100A4 into the extracellular space has been found to activate the NF-κB pathway and induce specific gene transcription (42). We have shown here that higher expression of S100A4 was directly correlated with stronger radioresistance of pancreatic cancer cell lines. These findings suggest that upregulation of S100A4 expression may contribute to the activation of the NF-κB pathway more strongly in pancreatic cancer cells, leading to a sequential increase in radioresistance via the transcription of genes associated with radioresistance. Taken together, these findings indicate that S100A4 increases radioresistance by interacting with transcription factors that induce radioresistance. Further investigations of the mechanisms underlying these interactions are required.

In summary, we showed that S100A4 expression in pancreatic cancer cell lines was augmented by continuous irradiation and that assaying S100A4 mRNA expression could predict the radioresistance of pancreatic cancer cell lines. Based on these findings, we conclude that S100A4 is involved in the radioresistance of pancreatic cancer cell lines and may be involved in the mechanism by which pancreatic cancer acquires radioresistance. Treatment with agents targeting S100A4, along with radiation, may improve the prognosis of patients with pancreatic cancer undergoing radiotherapy.

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