

The Proangiogenic Factor Ephrin-A1 Is Up-Regulated in Radioresistant Murine Tumor by Irradiation

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While the pre-treatment status of cancer is generally correlated with outcome, little is known about microenvironmental change caused by anti-cancer treatment and how it may affect outcome. For example, treatment may lead to induction of gene expression that promotes resistance to therapy. In the present study, we attempted to find a gene that was both induced by irradiation and associated with radioresistance in tumors. Using single-color oligo-microarrays, we analyzed the gene expression profiles of two murine squamous cell carcinomas, NR-S1, which is highly radioresistant, and SCCVII, which is radiosensitive, after irradiation with ¹³⁷Cs gamma rays or carbon ions. Candidate genes were those differentially regulated between NR-S1 and SCCVII after any kind of irradiation. Four genes, *EfnA1* (Ephrin-A1), *Sprr1a* (small proline-rich protein 1A), *Srgap3* (SLIT-ROBO Rho GTPase activating protein 3) and *Xrra1* [RIKEN 2 days neonate thymus thymic cells (NOD) cDNA clone E430023D08 3'], were selected as candidate genes associated with radiotherapy-induced radioresistance. We focused on *EfnA1*, which encodes a ligand for the Eph receptor tyrosine kinase known to be involved in the vascular endothelial growth factor (VEGF) pathway. We used immunohistochemical methods to detect expression of Ephrin-A1, VEGF, and the microvascular marker CD31 in radioresistant NR-S1 tumor cells. Ephrin-A1 was detected in the cytoplasm of NR-S1 tumor cells after irradiation, but not in SCCVII tumor cells. Irradiation of NR-S1 tumor cells also led to significant increases in microvascular density, and up-regulation of VEGF expression. Our results

suggest that radiotherapy-induced changes in gene expression related with angiogenesis might also modulate microenvironment and influence responsiveness of tumors. *Exp Biol Med* 234:112–122, 2009

Key words: Ephrin-A1; radioresistance; irradiation; mouse tumor model; microarray

Introduction

An estimated 60,000 new cases of cancer were diagnosed in Japan in 2003, and about 25% of these patients received some form of radiotherapy (1). Radiotherapy is one of the most important treatments for localized disease, and ionizing radiation is used to treat virtually all types of solid malignancies, with varying degrees of success.

Recently, carbon ion (C-ion) radiotherapy for solid tumors has come into use as a therapeutic treatment. C-ions have an excellent dose distribution and higher linear energy transfer than X-rays or gamma rays, and may produce higher therapeutic effects against cancer (2, 3). We have investigated the biological effects of C-ion radiation using microarray analysis in multiple murine tumor models, and found that C-ion and ionizing irradiation had largely similar effects on gene expression. Surprisingly, we found a few murine tumors were extremely resistant to both C-ion irradiation and even high doses of gamma rays (4). Clinical studies of the efficacy of C-ion radiotherapy have also encountered radioresistant tumors. For certain tumors such as non-small cell lung cancer, locally advanced head and neck squamous cell carcinoma and bone and soft tissue sarcoma, 3-year local control rates were 64%, 34% and 73%, respectively (5–7).

The response of tumor cells to radiation includes complex changes in gene expression affecting many processes including DNA repair, regulation of the cell

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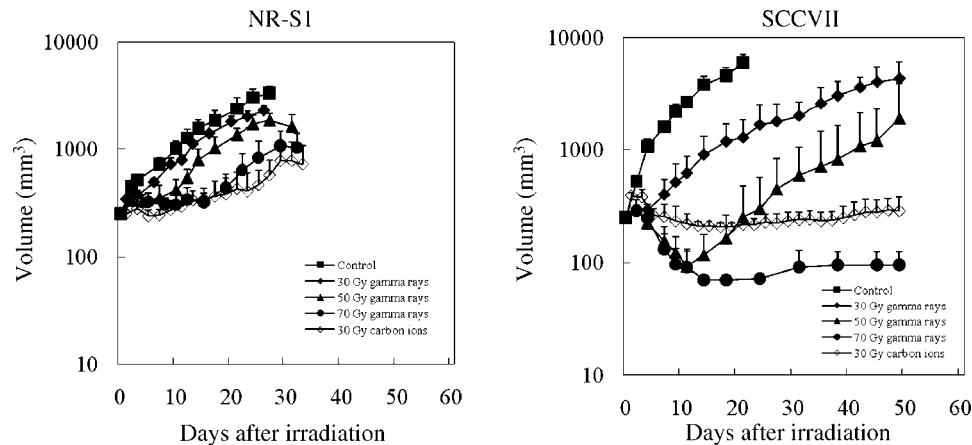


Figure 1. Growth curves for NR-S1 (left panel) and SCCVII (right panel) tumors in mice (5 mice each group). Tumor volume (mm^3) was measured every other day after sham irradiation (■), or a single dose of gamma rays at either 30 Gy (◆), 50 Gy (▲), or 70 Gy (●), or 30 Gy carbon ions (◇). The average tumor volume with SD (Supplementary Data SR3) was plotted against after irradiation. Results are expressed as mean $\pm$ SD. ($n = 5$).

cycle, cell proliferation, apoptosis, and growth metabolism (8–10). Cellular and genetic factors, including differential tissue-specific gene expression, are likely to underlie radiation-resistant cellular phenotypes (11). In addition, abundant studies have shown that tumor microenvironment, in particular tumor vasculature, is an important factor modulating tumor radiosensitivity (12–14).

However, most studies examining the tumor microenvironment have focused on the microenvironment prior to treatment. Little is known about whether radiotherapy itself might alter the tumor microenvironment, perhaps acting as a risk factor for tumor radioresistance.

In this study, we show that radioresistant murine squamous carcinoma tumors express increased levels of Ephrin-A1 and VEGF, and also have increased microvascular density, while radiosensitive tumors do not.

Material and Methods

Mice and Tumors. C3H/HeMsNrs male mice, aged 12–16 weeks ($n = 270$), were used in this study. The animals were produced and maintained in groups in specific

pathogen-free facilities in the National Institute for Radiological Sciences (NIRS). Two murine squamous cell carcinomas, NR-S1 and SCCVII, were used to induce tumor formation (4, 15–17). Single-cell suspensions were transplanted subcutaneously into the right hind leg of the mice (17). The study protocol was reviewed and approved by the NIRS Institutional Animal Care and Use Committee (protocol number 18–2015).

Irradiation. Tumor diameter was 7.5 ± 0.5 mm (mean $\pm$ range) at irradiation for all tumors. This size was reached 9 and 10 days after inoculation into the hind leg for NR-S1 and SCCVII tumors, respectively. Irradiation doses of C-ion used in this study were those sufficient to induce growth delay for local tumors in our previous study (4). Three grading doses of gamma rays were chosen: 1) 30 Gy, an equal physical dose to C-ion dose, 2) 70 Gy, a dose with equivalent biological effectiveness as the C-ion dose in a growth delay assay (4), and 3) 50 Gy, an intermediate dose.

Carbon-12 ions were accelerated by heavy-ion medical accelerator in Chiba (HIMAC) synchrotron up to 290 MeV/u (18). Exposures were conducted using horizontal carbon beams as a dose rate of approximately 3 Gy/min. The desired linear energy transfer (LET) was obtained by increasing a given thickness of polymethyl methacrylate upstream of the mice. Carbon beams with 50 keV/umLET were within the spread-out bragg peak (SOBP), as described elsewhere (19). Five mice were immobilized on a Lucite plate using pentobarbital anesthesia (50 mg/kg body weight) and the right hind legs were fixed in a rectangular field of 28×100 mm using tape. ^{137}Cs gamma rays with a dose rate of 1.3 Gy/min at a focus-skin distance of 21 cm were used as a reference beam. A doughnut-shaped radiation field with a 30 mm rim collimated the vertical beam for several mice per session. For irradiation, mice were subjected to a single dose irradiation using ^{137}Cs gamma rays or carbon-12 ions. Radiation was administered at 30, 50 and 70 Gy for gamma rays and at 30 Gy for carbon ions.

Table 1. Tumor Growth Delay (TGD) of Two Murine Tumors, NR-S1 and SCCVII

	TGD (days) ^a		<i>P</i> value ^c
	NR-S1	SCCVII	
30 Gy gamma rays	1	17	< 0.05
50 Gy gamma rays	9	38	< 0.05
30 Gy carbon ions	27	55 ^b	< 0.05

^a The difference between the tumor growth time (time required for each tumor to become 5-times as large as the initial volume) of an experimental group and that of a non-irradiated control, expressed as median.

^b After 50 days, five recurred tumors were observed in SCC tumors after 30 Gy carbon ion irradiation.

^c Wilcoxon signed-rank test.

The irradiation dose used was determined in preliminary experiments as the lowest dose needed to achieve tumor growth delay in this murine tumor model. Experimental models with spontaneous synergistic tumors required much higher irradiation doses than are used in the clinic. Pre-treatment control tumors were obtained from mice that had been anesthetized, but were not exposed to radiation.

Tumor Growth Delay Assay. Three diameters of each tumor were measured by a caliper every other day for up to 2 months after irradiation, and tumor volume was calculated as described previously (20). The tumor growth time, i.e., the time required for each tumor to become 5-times as large as the initial volume, was calculated from the first irradiation day, and the tumor growth times obtained for each animal were averaged per each treatment group. The difference between the tumor growth time of each treatment group and that of an unirradiated control was defined as the tumor growth delay (TGD) time (21). To obtain the TGD times, triplicate experiments were performed by using 5 mice per each treatment including previous report (4). TGD was used as the criteria for radiosensitivity.

Tissue Isolation. Animals were sacrificed either before irradiation (pre) or 1 day after irradiation (day 1) for histological investigation and transcriptome assays. Furthermore, for immunohistochemical analysis of CD31, tumors were extracted 15 days after irradiation. Whole tumors were removed immediately after sacrifice and divided into two sections. Half the tumor was fixed in 10% neutralized formalin and embedded in paraffin, and the other half was chopped and placed in RNAlater (Ambion, Austin, TX) and stored at -20°C . Tumor recurrence was followed for one month after growth inhibition. Recurring tumors, which regrew after complete inhibition of growth, were also extracted for histological investigation.

RNA Extraction, Probe Preparation, and Microarray Hybridization. Total RNA was purified using ISOGEN (Nippon Gene Co., Tokyo, Japan) followed by the RNeasy Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. RNA quality was verified by examining the integrity of 28S and 18S rRNA using the 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). Total RNA was pooled from tumors of 3 mice (3 tumors) per group. Double-stranded cDNA and subsequent cRNA was synthesized from 2 μg of total RNA using the CodeLink Expression Assay Kit (GE Healthcare Bio-Sciences Corp., Piscataway, NJ) according to the manufacturer's instructions (<http://www.codelinkbioarrays.com>). The bioarrays were single-color oligo-microarrays consisting of 44,000 mouse sequences (Mouse Whole Genome Array; GE Healthcare Bio-Sciences Corp.). They were stained with Cy5-streptavidin (GE Healthcare UK Ltd., Buckinghamshire, England) and scanned using a dual-laser microarray scanner (Agilent Technologies).

Analysis of Microarray Data. We examined all data from two arrays for each sample. Spot signals were

quantified using CodeLink Expression Analysis Software (Version 4.1; GE Healthcare Bio-Sciences Corp.). Output data was further analyzed using Resolver software (Version 5; Rosetta Biosoftware, Seattle, WA), described previously (4). All non-detection calls were filtered prior to performing the analysis. We averaged scan data from two replicate array experiments using an error-weighted algorithm. All array data were median-normalized by z-score transformation (22). Our raw data, including sample information, intensity measurements, error analysis, microarray content, and slide hybridization conditions are available at <http://www.ncbi.nlm.nih.gov/geo/> with the accession number GSE5331.

Histopathology. Formalin-fixed, paraffin-embedded samples of each tumor were sectioned at an average thickness of 3 μm and either stained with hematoxylin and eosin, or processed as follows. Sections were deparaffinized and Protease 1 (Ventana Medical Systems, Tucson, AZ) was added for 8 mins at 37°C . Immunohistochemical studies were performed to detect expression of Ephrin-A1, PECAM-1 (CD31) and vascular endothelial growth factor (VEGF) using a streptavidin-biotin immunoperoxidase technique as described elsewhere (23) and a Discovery XT automated immunohistochemistry processor (Ventana Medical Systems). Rabbit anti-Ephrin-A1 antibody (Zymed Laboratories, South San Francisco, CA), rat anti-CD31 antibody (BMA Biomedicals AG, Augst, Switzerland), and rabbit anti-VEGF antibody (Santa Cruz Biotechnology, Santa Cruz, CA) were diluted in antibody dilution buffer (Ventana Medical Systems) at 1:50, 1:100 and 1:100, respectively. For positive controls, we used human breast cancer tissue for Ephrin-A1, and normal mouse lung tissue for CD31 and VEGF. For negative controls, we followed the same procedure but omitted the primary antibody. Sections were photographed using a BX51 microscope (Olympus, Tokyo, Japan). CD31 staining was quantified by counting the number of CD31-positive vessels per $\times 200$ field in four random fields per section.

Quantitative RT-PCR. Total RNA from one tumor of one mouse (3 tumors per group) was used for qRT-PCR analysis, whereas total RNA from tumors of 3 mice (3 tumors) per group was pooled and subjected to microarray analysis. DNase-treated total RNA extracted from each tumor sample was reverse transcribed using the Transcriptor 1st Strand cDNA Synthesis Kit (Roche Diagnostics GmbH, Mannheim, Germany). Web-based array design software was used to design intron-spanning primer pairs and to select appropriate hybridization probes from the Universal ProbeLibrary (Roche). Quantitative RT-PCR (qRT-PCR) was performed on a LightCycler 480 Instrument (Roche) using the LightCycler 480 Probes Master (Roche) according to the manufacturer's recommendations. All samples were normalized to expression of the reference gene, *Naca*, which displays minimal expression variation in murine pre-irradiated and irradiated tumors (4), and relative values were plotted in comparison to gene expression from pre-

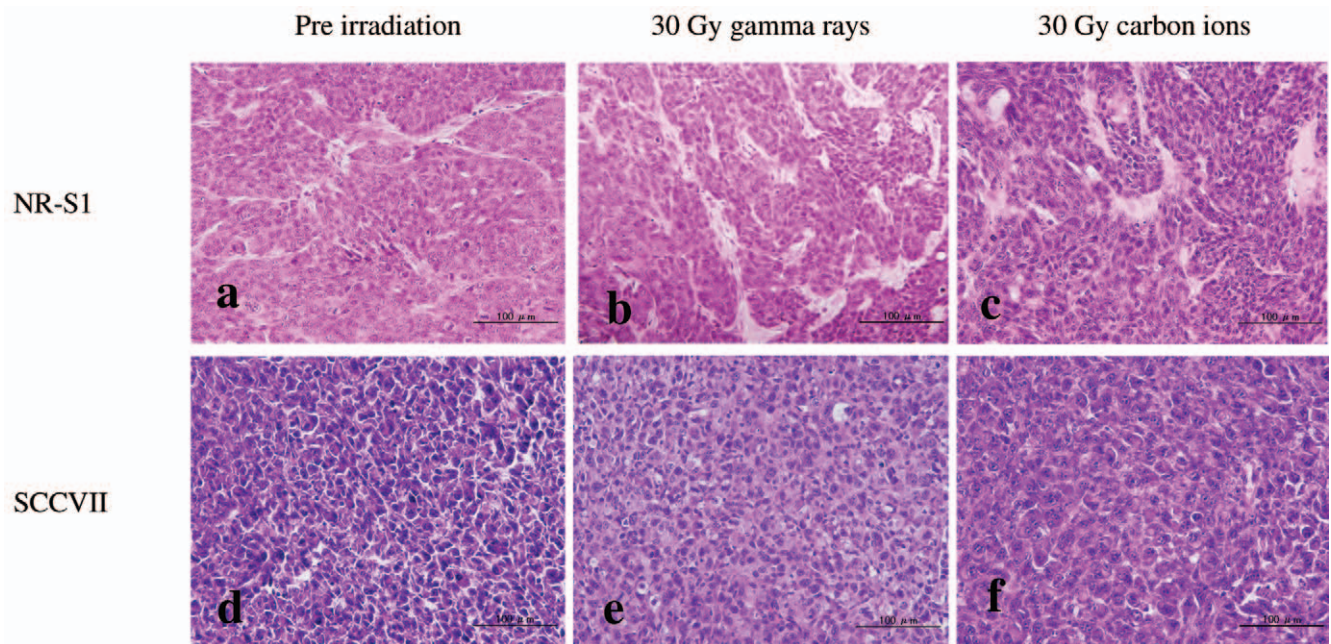


Figure 2. Hematoxylin and eosin-stained sections of NR-S1 (upper panel) and SCCVII (lower panel) tumor samples: (a) NR-S1 pre-irradiation, (b) NR-S1 irradiated with 30 Gy gamma rays at 1 day after irradiation, (c) NR-S1 irradiated with 30 Gy carbon ions at 1 day after irradiation, (d) SCCVII pre-irradiation, (e) SCCVII irradiated with 30 Gy gamma rays at 1 day after irradiation, (f) SCCVII irradiated with 30 Gy carbon ion at 1 day after irradiation. Magnification $\times 400$. A color version of this figure is available in the online journal.

irradiated tumors. Primer sequences are: *Efnal*-forward: 5'-GGAACAGTTCAAATCCCAAGTT-3', reverse: 5'-CTCGTAATGTGGGCAGATGAT-3'; *Naca*-forward: 5'-ACAAAATGCCCGGTGAAG-3', reverse: 5'-CA-GACTCTGTTCCCGATCCT-3'.

Statistical Analysis. Unpaired Student's *t* tests were performed to detect differences between the ratio values of gene expression in the two tumor types, NR-S1 and SCCVII. *P* values of less than 0.05 were considered statistically significant. Tumor growth delay was compared by Wilcoxon signed-rank test and the number of CD31 positive blood vessels in different samples were compared by Mann-Whitney *U* tests.

Results

Tumor Growth Inhibition. Both NR-S1 and SCCVII tumors displayed some growth inhibition in

response to 70 Gy gamma rays and 30 Gy carbon ion irradiation (Fig. 1). However, SCCVII tumors were significantly more sensitive to radiation with 30 Gy and 50 Gy gamma rays, and 30 Gy carbon ions than NR-S1 tumors (Fig. 1). After 50 days, five recurred tumors were observed in SCC tumors after 30 Gy carbon irradiation and no recurred tumors were observed after 70 Gy gamma irradiation. When we calculated the time required for each tumor to become 5-times as large as the initial volume, and assessed tumor growth delay (TGD), there was a statistically significant difference between two tumors. NR-S1 tumors were more radioresistant than SCCVII tumors (Table 1). An assessment of tumor growth delay (TGD) reveals that NR-S1 tumors are more radioresistant than SCCVII tumors (Table 1). SCCVII, which displayed a strong radiosensitive phenotype, was used as a reference tumor to evaluate changes in expression of candidate genes in response to irradiation.

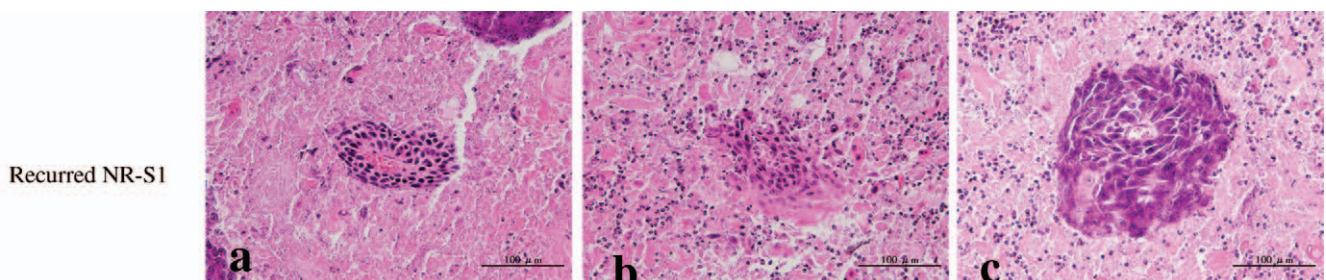


Figure 3. Hematoxylin and eosin-stained sections of recurred NR-S1 tumor samples 20 days after carbon ion irradiation. Magnification $\times 400$. A color version of this figure is available in the online journal.

Tumor Histology. Histology was performed on NR-S1 and SCCVII tumor samples before and after irradiation to examine the cytological effects of gamma and C-ion radiation. Prior to irradiation, NR-S1 tumors consisted of well to moderately differentiated squamous carcinoma cells with fibrous pseudocapsules, and smudged chromatin and bizarre mitotic figures were evident (Fig. 2a). SCCVII pre-irradiated tumors consisted of poorly differentiated squamous carcinomas comprised of sarcomatous cells, and loss of intercellular adhesions was frequently observed (Fig. 2d). On day 1 after 30 Gy gamma irradiation, SCCVII tumor cells (from tumors categorized as radiosensitive according to TGD results) showed evidence of cellular damage including pyknosis, karyorrhexis and slight swelling of the nuclei and cytoplasm (Fig. 2e). In contrast, NR-S1 cells (from tumors categorized as radioresistant) showed less evidence of cytotoxic effects (Fig. 2b). C-ion irradiation induced histological changes in both SCCVII and NR-S1 tumors similar to those resulting from gamma irradiation (Fig. 2c, 2f). Recurring NR-S1 tumors, which re-grew as palpable tumors in the primary sites, were examined histologically at day 20 after C-ion irradiation. Recurring tumors contained eosinophilic effusion with infiltrating inflammatory cells, and clonogenic tumor colonies lined capillaries (microvessels) as shown in Figure 3.

Identification of Genes Responding to Gamma Rays and Carbon Ions. Gene expression in NR-S1 and SCCVII tumors, before and after irradiation, was examined using a mouse whole genome microarray as described in Materials and Methods. We first selected potential genes associated with radioresistance at three doses of gamma irradiation as follows: 1) by searching for genes up-regulated in NR-S1 tumors and down-regulated in SCCVII tumors, and vice versa, after irradiation, 2) by looking for genes with a significant difference in their ratio values for NR-S1 versus SCCVII tumors ($P < 0.05$, unpaired Student's t test), and 3) by comparing the expression values of genes before and after irradiation using a filter for at least 1.5-fold change. Eight genes were induced or repressed > 1.5 -fold change in gamma ray irradiated tumor relative to pre-irradiated control tumor (Table 2). Of these, only *Efnal* (ephrin A1), *Sprrla* (small proline-rich protein 1A), *Srgap3* (SLIT-ROBO Rho GTPase activating protein 3) and *Xrral* [RIKEN 2 days neonate thymus thymic cells (NOD) cDNA clone E430023D08 3'] were expressed differently in resistant (NR-S1) and sensitive (SCCVII) tumors after carbon ion irradiation (Fig. 4). *Efnal* is a novel candidate gene that might be useful in predicting responsiveness to radiation, while *Xrral* (X-ray radiation resistance associated 1) was previously identified as a radio-resistant factor (24).

Confirmation of Differential *Efnal* Expression by Quantitative RT-PCR. We examined *Efnal* mRNA levels in NR-S1 and SCCVII tumors following gamma and carbon ion irradiation using quantitative RT-PCR. Expression levels were normalized to the expression of the reference gene, *Naca*, which shows minimal expression

Table 2. Eight Selected Genes Differentially Regulated in Two Tumors (NR-S1 and SCCVII) After Gamma Irradiation

Gene name	Description	Accession number	NR-S1 (fold change ^a)			SCCVII (fold change)		
			30 Gy	50 Gy	70 Gy	30 Gy	50 Gy	70 Gy
<i>Efnal</i>	Ephrin A1 (<i>Efnal</i>)	NM_010107.2	1.6	2.2	1.8	-1.5	-1.8	-2.1
<i>Mal</i>	Myelin and lymphocyte protein, T-cell differentiation protein (<i>Mal</i>)	NM_010762.2	2.0	3.8	3.5	-3.0	-1.8	-2.0
<i>Mst1</i>	Macrophage stimulating 1 (hepatocyte growth factor-like) (<i>Mst1</i>)	NM_008243.2	2.3	3.3	2.7	-3.9	-3.5	-3.1
<i>Pycard</i>	PYD and CARD domain containing (<i>Pycard</i>)	NM_023258.3	2.0	1.9	2.4	-1.5	-1.6	-1.7
<i>Sprrla</i>	Small proline-rich protein 1A (<i>Sprrla</i>)	NM_009264.1	2.4	5.4	8.1	-1.7	-1.6	-7.7
<i>Srgap3</i>	SLIT-ROBO Rho GTPase activating protein 3 (<i>Srgap3</i>)	NM_153070.1	1.7	2.2	2.6	-1.6	-1.7	-2.6
<i>Xrra1</i>	RIKEN 2 days neonate thymus thymic cells (NOD) cDNA clone E430023D08 3'	BY762892.1	-3.6	-4.6	-4.4	3.9	2.1	4.4
<i>Tnn</i>	Tenascin N (<i>Tnn</i>)	NM_177839.2	-3.5	-2.9	-1.7	3.7	1.5	1.9

^a Fold change was calculated from the intensities of irradiated samples versus pre-irradiated samples.

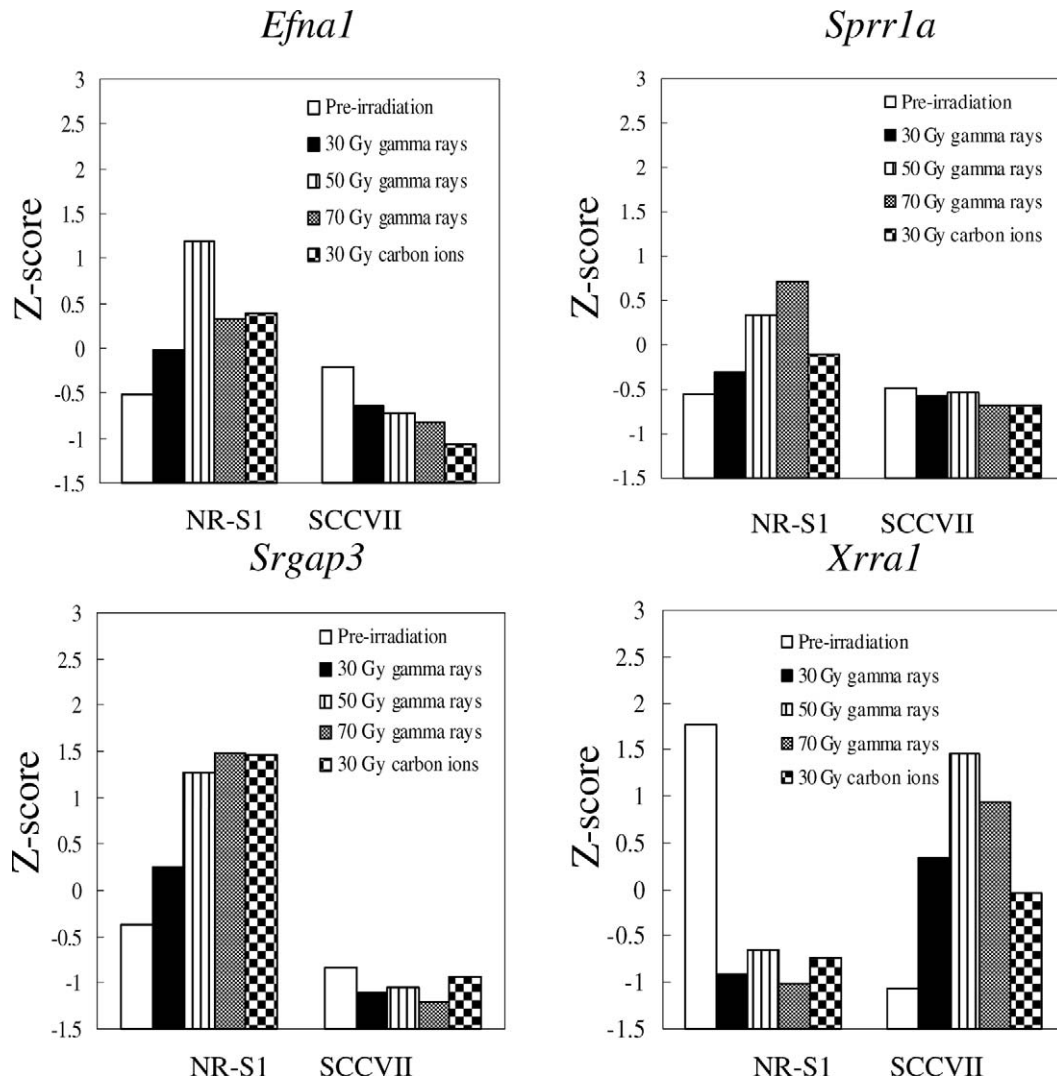


Figure 4. Z-score (expression intensity) of *Efnal* (upper left), *Sprr1a* (upper right), *Srgap3* (lower left) and *Xrra1* (lower right panel). Expression intensity pre-irradiation (open bars), one day after 30 Gy gamma irradiation (closed bars), 50 Gy gamma irradiation (striped bars), 70 Gy gamma irradiation (cross-hatched bars), or 30 Gy carbon ion irradiation (stippled bars) was evaluated by microarray analysis of NR-S1 and SCCVII tumor samples.

variation in pre-irradiated and irradiated tumors. The relative values were plotted in comparison to the corresponding pre-irradiated tumor sample. *Efnal* was up-regulated in NR-S1 and down-regulated in SCCVII after both gamma and carbon ion irradiation (Fig. 5A, B).

Immunohistochemical Analysis for Ephrin-A1. We also examined Ephrin-A1 protein levels in NR-S1 and SCCVII tumor cells before and after irradiation. Ephrin-A1 was not detected in either NR-S1 or SCCVII tumor cells before irradiation (Fig. 5C-a, d). One day after gamma irradiation, Ephrin-A1 was detected in the cytoplasm of tumor cells in the radioresistant NR-S1 tumors (Fig. 5C-b), but not in the radiosensitive SCCVII tumors (Fig. 5C-e). Ephrin-A1 was also strongly expressed in NR-S1 tumor cells after carbon ion irradiation (Fig. 5C-c), but not detected in SCCVII tumor cells (Fig. 5C-f).

Immunohistochemical Analysis for CD31. As tumor microenvironment, in particular tumor vasculature, is an important factor modulating tumor radiosensitivity and Ephrin-A1 is a known proangiogenic factor, we examined whether microvascular density was increased in the NR-S1 tumors by staining sections for expression of the capillary marker protein CD31 (25). When we investigated microvascular density at 15 days after gamma irradiation, significant increases in microvascular density were observed in radioresistant NR-S1 tumors relative to pre-irradiated controls (Fig. 6A and 6B-a, b). CD31 was also expressed in NR-S1 tumors after carbon ion irradiation (Fig. 6B-c).

Immunohistochemical Analysis for VEGF. Because Ephrin-A1 is known to up-regulate expression of the angiogenesis factor VEGF (25), we examined VEGF expression in the NR-S1 radioresistant tumors. VEGF

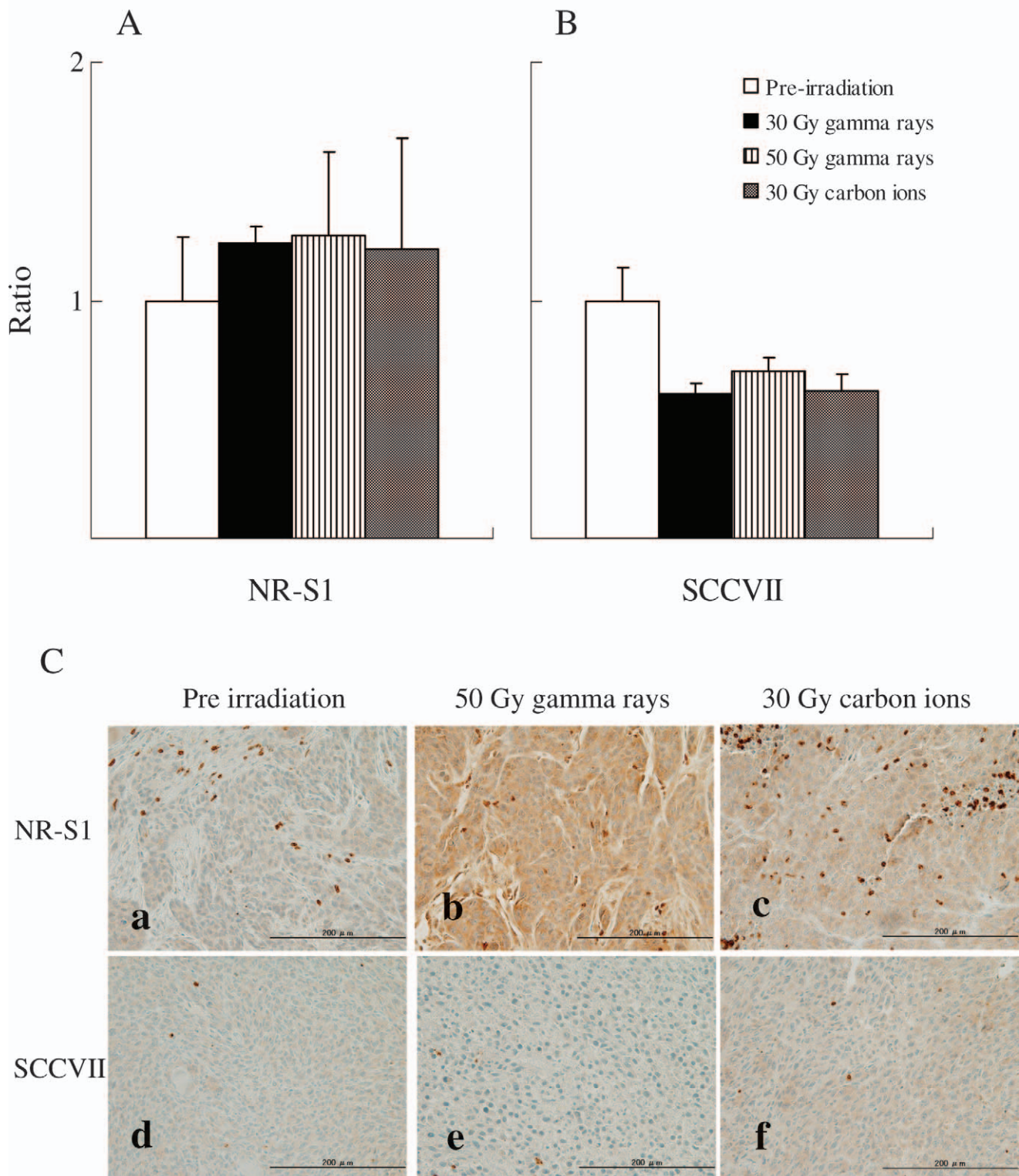
Efna1

Figure 5. Quantification of *Efna1* mRNA. All samples were analyzed by qRT-PCR and mRNA levels were normalized to the expression of the reference gene, *Naca*, which shows minimal expression variation in pre-irradiated and irradiated tumors (4). (A) The relative values are plotted in comparison to the corresponding pre-irradiated NR-S1 tumor sample and data are expressed as ratios (mean $\pm$ standard deviation). (B) Those are plotted in comparison to the corresponding pre-irradiated SCCVII tumor sample. Results displayed are one day after 30 Gy gamma irradiation (closed bars), 50 Gy gamma irradiation (striped bars), or 30 Gy carbon ion irradiation (stippled bars). (C) Immunohistochemistry of Ephrin-A1 in NR-S1 (upper) and SCCVII (lower) tumor samples: (a) NR-S1 pre-irradiation, (b) NR-S1 one day after 50 Gy gamma irradiation, (c) NR-S1 one day after 30 Gy carbon ion irradiation, (d) SCCVII pre-irradiation, (e) SCCVII one day after 50 Gy gamma irradiation, and (f) SCCVII one day after 30 Gy carbon ion irradiation. Magnification $\times 400$. A color version of this figure is available in the online journal.

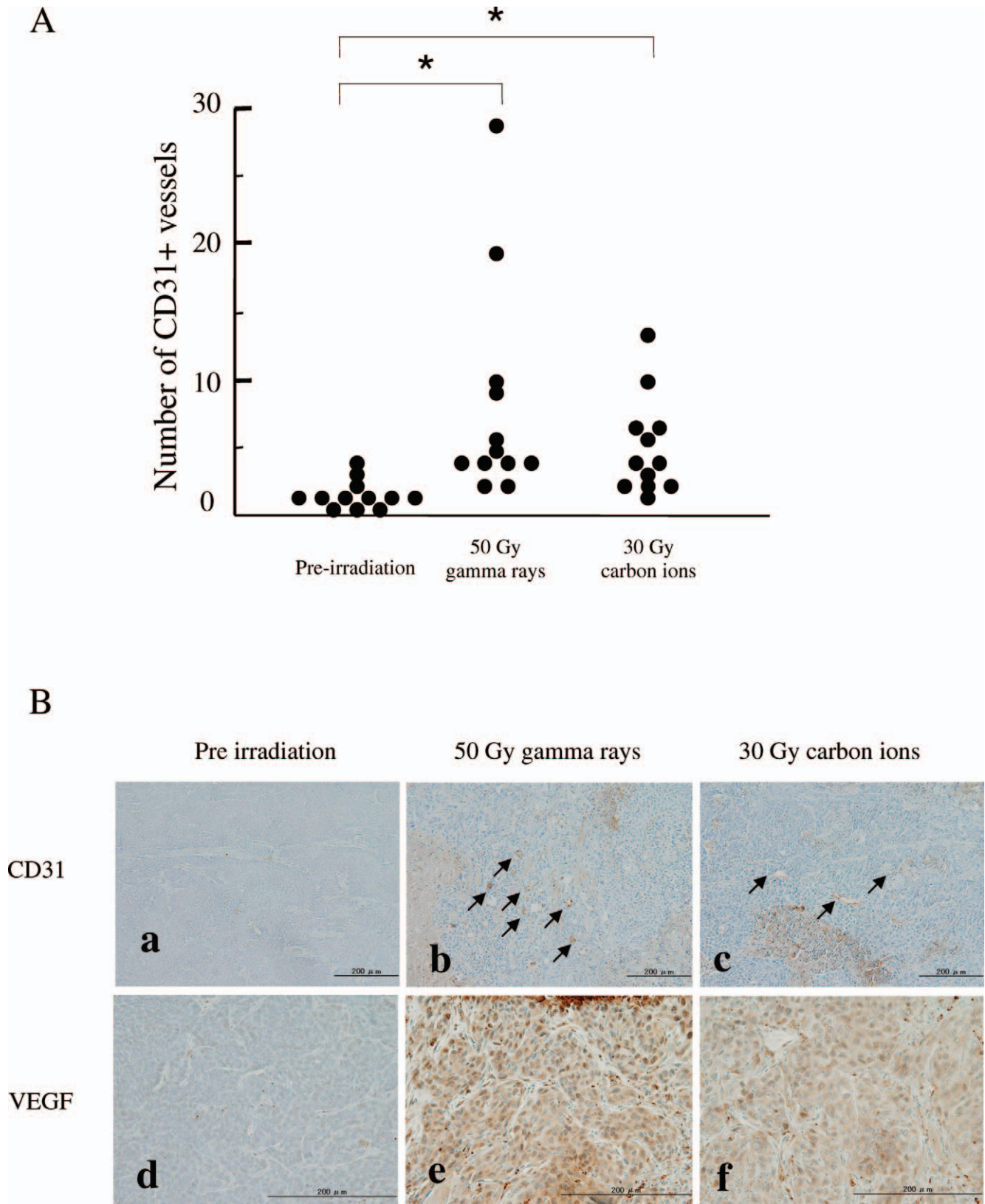


Figure 6. Immunohistochemical expression of CD31 and VEGF. (A) Number of CD31 positive (+) blood vessels in NR-S1 tumor samples pre-irradiation and 15 days after 50 Gy gamma irradiation or 30 Gy carbon ion irradiation. * Significant difference between groups ($P < 0.05$, $n = 3$ independent tumors). (B) Representative immunohistochemical expression of CD31 (upper) and vascular endothelial growth factor (VEGF, lower panel). (a–c) Sections stained for CD31 from NR-S1 tumors pre-irradiation, 15 days post-irradiation with 50 Gy gamma rays or 30 Gy carbon ions respectively. Arrows indicate CD31+ tumor blood vessels. Magnification $\times 200$. (d–f) VEGF staining in NR-S1 tumors pre-irradiation, and one day post-irradiation with 50 Gy gamma rays or 30 Gy carbon ions, respectively. Magnification $\times 400$. A color version of this figure is available in the online journal.

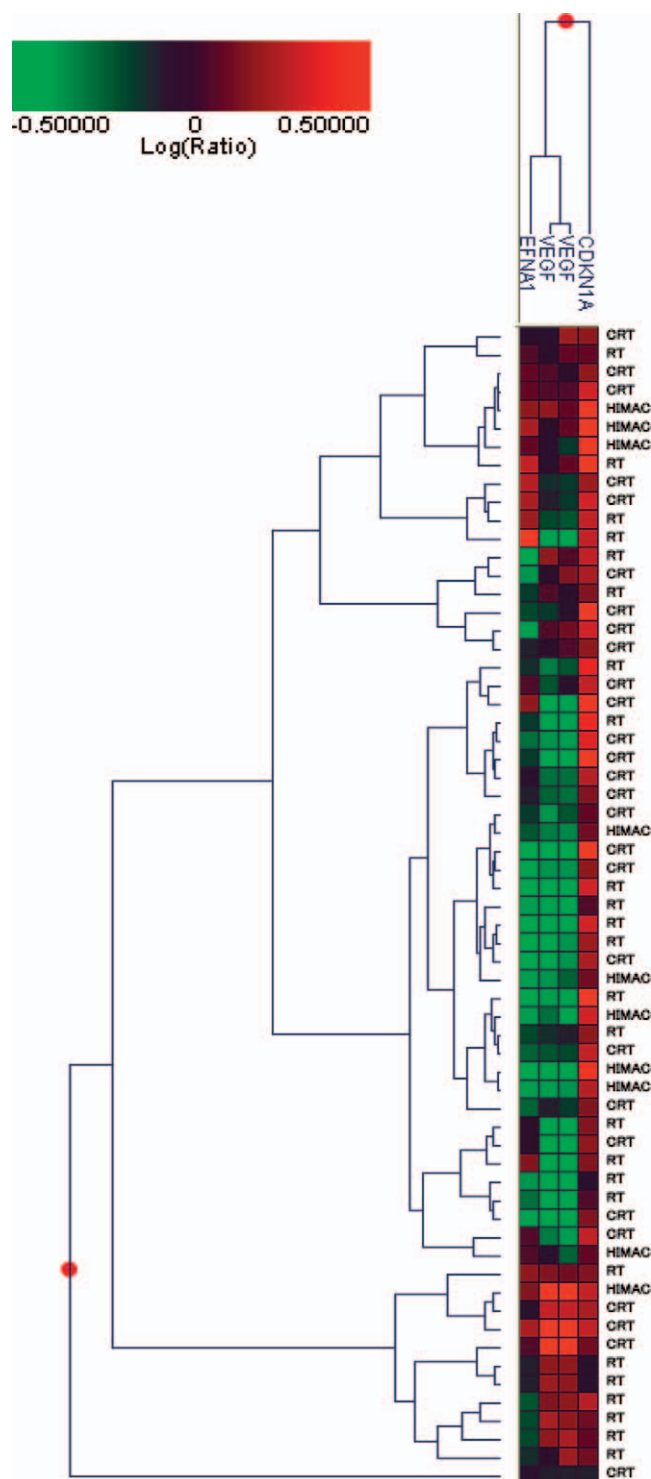


Figure 7. Two-dimensional hierarchical clustering of *Efnal*, *VEGF* and *ckdnl1a* expression changes in 63 cervical cancer patients (34). Changes in gene expression induced by radiation treatment were investigated using single-color oligo-microarrays consisting of 44,000 human sequences. Ratio expression changes were calculated by pretreatment and midtreatment samples. A second biopsy (midtreatment) was taken 1 week after the start of therapy, as described elsewhere (34). At that time, patients in the chemoradiotherapy (CRT) and radiotherapy (RT) groups had received 9 Gy of whole pelvic irradiation, and the CRT group had also received a single dose of cisplatin, but brachytherapy had not yet been started. Patients in

protein was not detected before irradiation (Fig. 6B-d), but was detected in the cytoplasm of NR-S1 tumor cells one day after both gamma and carbon ion irradiation (Fig. 6B-e, f).

Discussion

To begin to examine whether radiation treatment may cause microenvironmental changes that might affect treatment outcomes, including radiation resistance, we have identified genes that are differentially expressed in murine tumors following local gamma or carbon ion irradiation including *Xrral*, *Sprrla*, *Srgap3* and *Efnal*.

Microarray analysis of gene expression revealed that *Xrral* was down-regulated, and *Sprrla*, *Srgap3* and *Efnal* were up-regulated, in radioresistant NR-S1 tumors after both gamma and carbon ion irradiation. In radiosensitive SCCVII tumors, in contrast, *Sprrla*, *Srgap3* and *Efnal* were down-regulated, and *Xrral* was up-regulated (Fig. 4). We focused on *Efnal*, which encodes a ligand for the Eph receptor tyrosine kinase, for several reasons. First, *Efnal* expression was significantly changed after gamma and carbon ion irradiation in both tumor types. Second, this gene was not previously known to be associated with a radiation response or radiosensitivity, and third, several studies suggest that *Efnal* is important in multiple aspects of tumorigenesis, including abnormal cell growth, invasion, metastasis, and especially angiogenesis (26–32). We confirmed the altered expression of *Efnal* in tumor cells following radiation by quantitative real-time PCR and immunohistochemistry (Fig. 5). We then demonstrated by immunohistochemical studies that VEGF expression and microvascular density are both increased in the NR-S1 tumors (Fig. 6).

With respect to tumor angiogenesis, *Efnal* has proangiogenic effects through at least two distinct mechanisms: (a) activating the EphA2 receptor on host blood vessel endothelial cells, and (b) up-regulating VEGF expression in tumor cells and subsequent activation of endothelial cells (25). Expression analysis of several mouse xenograft models and human tumors indicates that *Efnal* is widely expressed in tumor parenchyma and tumor endothelium (28). VEGF has been shown to enhance endothelial cell survival and VEGF-positive xenografts are more resistant to the cytotoxic effects of ionizing radiation (33). In this study, *Efnal* was induced by both ionizing radiation and charged particles (carbon ions) in the radioresistant tumors and VEGF expression was induced in NR-S1. Furthermore, we observed recurring tumor cells lining microvessels (Fig. 3), suggesting that neoangiogenesis is important for tumor recurrence.

← the carbon-ion radiotherapy (HIMAC) group had received 12.5 GyE for local tumors. All specimens were excised from the same site in the cervical tumors, before radiation therapy and at 6 hrs after the fifth irradiation. A color version of this figure is available in the online journal.

Experimental models with spontaneous synergistic tumors often require much higher irradiation doses than are used in the clinic, and doses of similar intensity have been used in other studies done in mouse tumor models (21). Based on our own preliminary experiments, we selected a single dose of 30 Gy of carbon ion beams and 30–70 Gy of gamma rays, which gave significant growth delay in murine tumors. In this selection of irradiation doses, the local tumor effect got the priority over conventional experimental doses, such as 2 Gy.

In our preliminary research with clinical samples, which were sequentially taken from cervical cancer patients, concomitant activation of *VEGF* with *Efnal* was observed in clinical samples from patients with cervical cancer, with increased expression following radiation therapy (Fig. 7). Biopsy specimens were obtained before and during radiotherapy, and changes in gene expression induced by treatment were investigated using microarray (34). Although it is still too early to evaluate the predictive value of *Efnal*, as the follow-up time is not enough in this clinical setting, these findings underscore the potential importance of treatment-induced changes in the tumor microenvironment, and further implicate the VEGF and Ephrin-A1 pathways in radioresistance. VEGF antibodies, such as bevacizumab, and several new tyrosine kinase inhibitors targeting the VEGF pathway, such as gefitinib and zactima, are being pursued as promising targeted molecular therapies, especially in combination with radiotherapy (35–37). Our results suggest that the Ephrin-A1/EphA2 pathway might be another avenue for targeted therapy for radioresistant solid tumors.

The *Xrral* gene (X-ray radiation resistance associated 1) was recently identified by Mesak and colleagues as being down-regulated (two-fold) in an X-ray radiation resistant cell clone, HCT116^{Clone2_XRR}, and associated with radioresistance in *in vitro* studies (24). In our study, *Xrral* was also down-regulated in radioresistant NR-S1 tumors, and up-regulated in radiosensitive SCCVII tumors. *Sprrla* is up-regulated after ultraviolet irradiation of epithelial cells (38). We therefore need further investigation of the roles of *Sprrla* and *Srgap3* in the response to radiation.

In this study, we did not focus on the difference between gamma and carbon ion irradiation. Our previous study suggested that some of carbon ion-induced genes, such as *Ikbke*, show specific expression changes (4). *Ikbke*/*IKKi*/*Ikkc* is a key integrator of signaling induced by pro-inflammatory stimuli, and for functional coordination between C/EBP and NFκB pathways during the immune response (39). These pathways may provide another mechanism underlying the biological effectiveness of carbon ion irradiation, and further investigation should be still necessary.

In conclusion, we have identified a number of genes that are up-regulated after both gamma and carbon ion irradiation in radioresistant NR-S1 murine tumors, including *Efnal*, a proangiogenic factor. Our data suggest that

radiotherapy-induced changes in expression of angiogenesis factors in radioresistant tumors might modulate the tumor microenvironment and influence responsiveness of tumors. Therapeutic combination of radiotherapy with biological modifiers targeting these molecules might improve therapeutic outcome.

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