Tumorigenesis and Neoplastic Progression

Radiation-Induced Increase in Cell Migration and Metastatic Potential of Cervical Cancer Cells Operates Via the K-Ras Pathway

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Radiotherapy is a well established treatment for cervical cancer, the second most common cancer in women worldwide. However, metastasis often circumvents the efficacy of radiotherapy. This study was conducted to elucidate the molecular mechanism of radioresistance-associated metastatic potential of cervical cancer cells. We established three radioresistant cervical cancer cell lines by exposure of cells to a sublethal dose of radiation and screened for lines that exhibited an increased migration phenotype for at least 6 months before undertaking mechanistic studies. Radiation-associated metastatic potential was evaluated using a wound-healing assay, time-lapse recording, and cell locomotion into the lungs of BALB/c nude mice. The radioresistant C33A and CaSki cell lines, but not the radioresistant HeLa cell line, exhibited significantly increased cell migration and wound healing than did wild-type cells. Furthermore, K-Ras played a prometastatic role via the activation of c-Raf/p38, whereas interference of those mediators via either RNA interference-mediated knockdown or the use of chemical inhibitors substantially reversed the radioresistance-associated increase in cell migration. Clinical examination further showed the relative upregulation of the K-Ras/c-Raf/p38 pathway in locally recurring tumors and distant metastases compared with in the primary cervical tumor. These findings demonstrate that a sublethal dose of radiation can enhance the metastatic potential of human cervical cancer cells via K-Ras/c-Raf/p38 signaling, highlighting the potential development of specific inhibitors for reducing metastatic potential during radiotherapy. (Am J Pathol 2012, 180:862–871; DOI: 10.1016/j.ajpath.2011.10.018)

Cervical cancer is the second most common cancer in women worldwide, and radiotherapy is an established treatment method.1 Although the cure rate of cervical cancer using radiotherapy is high, its failure to cure is frequently associated with metastasis.2,3 However, the mechanism of this radiation-associated metastatic potential of cervical cancer remains largely elusive.

The Ras subfamily of small GTPases has also been demonstrated to mediate radioresistance in a rodent model system4–6 and in several cell types.7,8 Recent evidence has also shown that Ras proteins play a critical role in invasion and metastasis in various cell types.9–13 The best-characterized effector proteins of Ras are the Raf serine/threonine kinases, such as c-Raf, activation of which leads to phosphorylation of the mitogen-activated protein kinase (MAPK) family.14 The MAPK family is classified into three groups: the extracellular signal–regulated protein kinases (ERK/MAPK subfamily), the p38 MAPKs, and the c-Jun N-terminal kinases (JNKs).15 The MAPKs have well-established roles in cell proliferation, differentiation, inflammation, and oncogenesis, and recent evidence also indicates their involvement in cell migration. For example, JNK is involved in cell migration via phosphorylation of paxillin and microtubule-associated proteins such as MAP1B, MAP2, and doublecortin in neural cells.16–18 p38 modulates migration by phosphorylating MAPK-activated protein kinase 2/3, which seems to be important for directionality of migration in smooth muscle

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cells and endothelial cells. ERK governs cell movement by activating integrin or phosphorylating myosin light chain kinase or focal adhesion kinase. However, whether the Ras-mediated MAPK signaling pathways are involved in radioresistance-enhanced metastatic potential in cervical cancer and the underlying mechanisms remain largely unknown.

Previous studies showed that a significant proportion of patients who underwent radiation treatment for cervical cancers subsequently developed metastasis. In that regard, we were interested in clarifying whether a Ras-mediated MAPK pathway may underlie the radiation-induced metastatic potential of human cervical cancer cells. To address this issue, we established three radioresistant cervical cancer cell lines from HeLa, C33A, and CaSki cells by exposing them to a sublethal dose of radiation. We found that the radioresistant C33A and CaSki cell lines exhibited an increased migration phenotype for at least 6 months. The capability depended on increased K-Ras activation mediated mainly through the phospho–c-Raf/p38 signaling cascade. We also validated the metastatic potential using an in vivo nude mouse model with tail vein injection of wild-type or radioresistant CaSki cells. Clinical specimens also showed relative increased activation of the K-Ras/c-Raf/p38 pathway in locally recurring and metastatic tumor cells compared with in the primary cervical tumor. This work may provide the basis for developing specific inhibitors to reduce metastatic potential during radiotherapy for cervical cancers.

Materials and Methods

Antibodies and Reagents

The following primary antibodies were obtained from Cell Signaling Technology Inc. (Beverly, MA): Ras (27H5) rabbit monoclonal antibody, c-Raf antibody, phospho–c-Raf (Ser338) antibody, p44/42 MAPK (Erk1/2) antibody, phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (E10) mouse monoclonal antibody, p38 MAPK antibody, phospho-p38 MAPK (Thr180/Tyr182) antibody, SAPK/JNK antibody, and phospho-SAPK/JNK (Thr183/Tyr185) antibody. Antibodies against K-Ras, H-Ras, and β-actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Epidermal growth factor receptor (EGFR) rabbit monoclonal antibody was obtained from Epitomics Inc. (Burlingame, CA). Hoechst 33342; small-interfering RNAs (siRNAs) targeting mRNAs encoding K-Ras, H-Ras, and c-Raf; and the transfection reagent Lipofectamine 2000 were purchased from Invitrogen (Carlsbad, CA). Dulbecco’s modified Eagle’s medium, RPMI 1640 medium, Opti-MEM reduced serum medium, fetal bovine serum, and antibiotics were purchased from Invitrogen. Selective inhibitors SB203580, U0126, and SP600125 and anti-auto-fluorescence reagents sodium borohydride and Sudan black were purchased from Sigma-Aldrich (St. Louis, MO). Epitope retrieval solution was purchased from Dako/mtm laboratories (Heidelberg, Germany). Fluorescein isothiocyanate–conjugated anti-mouse IgG, Alexa Fluor 488–conjugated goat anti-rabbit antibodies, Alexa Fluor 546–conjugated goat anti-mouse antibodies, and the CellTracker fluorescent cell-tracking dyes Green CMFDA and Orange CMRA were purchased from Invitrogen.

Patients and Tumor Materials

Paired tumor specimens were obtained from seven patients with cervical cancer who underwent radiotherapy at the Department of Radiation Oncology, Chang Gung Memorial Hospital–Kaohsiung Medical Center, Kaohsiung, Taiwan. Cervical tumor tissue specimens were collected before radiotherapy (primary tumor) and after radiotherapy (locally recurring tumors and distant metastases). The histopathologic diagnosis and tumor assessment criteria were classified and confirmed by pathologists. Retrospective clinical data collection and tissue specimen procurement were in accordance with the local guidelines for research ethics.

Radioresistant Cell Lines

HeLa, C33A, and CaSki human cervical carcinoma cell lines were obtained from the American Type Culture Collection (Manassas, VA). The culture conditions and media used were those stated in the American Type Culture Collection directory. Subconfluent cells were cultured at 37°C under an atmosphere of 5% CO₂ in a T25 flask. The irradiation protocol has been described elsewhere. Briefly, cells were cultured to 80% confluence and then were subjected to 6 Gy of radiation at a rate of 4 Gy per minute using a linear accelerator (Varian Medical Systems, Palo Alto, CA). Cells were kept at room temperature for <30 minutes during irradiation. One set of flasks for each cell type was not irradiated (wild type). After 24 hours of incubation, the medium in each flask was exchanged for fresh medium to remove detached cells. Cells resistant to radiation were propagated in the same medium, which was exchanged for fresh medium every 3 days thereafter. To generate stable radioresistant clones, all vital colonies were expanded for ≥180 days to confirm the radioresistant phenotype before studies were undertaken. Two clones from HeLa cells, three from C33A cells, and three from CaSki cells were established. The parental cells of wild-type stable clones were generated under the same conditions without irradiation.

Western Blot Analysis

Equal amounts of proteins were loaded into each lane, separated by SDS-PAGE, and then transferred onto a polyvinylidene difluoride membrane (Millipore, Billerica, MA) according to the standard procedure. Membranes were incubated with a primary antibody overnight at 4°C, followed by incubation with a secondary antibody and detection by enhanced chemiluminescence (PerkinElmer Inc., Waltham, MA). Membranes were then stripped and reprobed with antibodies against housekeeping gene β-actin (as internal control).
siRNA transfection was performed as described elsewhere. Briefly, siRNAs against human c-Raf, K-Ras, and H-Ras and corresponding guanine/cytosine content–matched scrambled siRNAs (negative controls) were individually transfected into cells at a final concentration of 40 nmol/L. Oligonucleotides were delivered to the cells using Lipofectamine 2000.

Wound-Healing Assay

Cells were cultured as confluent monolayers, synchronized in the absence of serum for 24 hours, and wounded by removing a 300- to 400-μm-wide strip of cells across the well using a standard 10-μL pipette tip. After washing, wounded regions were allowed to heal for 8 hours in serum-free medium before analysis. Wound healing was quantified as the ratio of the remaining cell-free area to the area of the initial wound (calculated as a percentage) using the public domain software ImageJ (http://rsbweb.nih.gov/ij/index.html).

Time-Lapse Recording of Cell Movement

Time-lapse recording of cell migration was performed as described elsewhere. Briefly, cells from the different conditions were seeded into a 6-well plate (1 × 10⁵ cells per well). Time-lapse recording was performed hourly using a microscope equipped with a camera system (Axiovert 200; Carl Zeiss MicrolImaging GmbH, Welwyn Garden City, UK). Phase-contrast images, visualized using the computer software supplied with the microscope system (AxioVision), were subjected to semiautomated tracing of cell migration.

Clonogenic Survival Curve Assay after Irradiation

A clonogenic assay was performed as described elsewhere. Briefly, subconfluent cells were cultured in a T25 flask with recommended medium and antibiotics at 37°C under an atmosphere of 5% CO₂. Cells (100 to 10,000 per well, according to the radiation dose) were seeded in 6-well plates immediately after irradiation. After 6 to 12 days, glutaraldehyde (6.0% v/v) and crystal violet (0.5% w/v) were added to fix and stain colonies, respectively. Colonies were counted using a stereomicroscope. Colonies consisting of ≥50 cells were counted as clonogenic survivors.

Immunofluorescence Cytochemical Staining of Phospho–c-Raf

Wild-type or radioresistant CaSki cells were transfected with siRNA for c-Raf for 48 hours and then were subjected to the wound-healing assay. After 8 hours, the coverslips were fixed, permeabilized, and stained for immunofluorescence analyses as described elsewhere. Briefly, permeabilized cells were washed three times with PBS and then were incubated at room temperature for 30 minutes with fluorescein isothiocyanate–conjugated anti-mouse IgG with PBS containing 2 mg/mL of bovine serum albumin. After washing three times with PBS, the coverslips were mounted in 1,4-diazabicyclo[2.2.2]octane (DABCO)/glycerol/PBS. The cells were visualized under a fluorescence microscope.

Double Immunofluorescence Labeling of Formalin-Fixed, Paraffin-Embedded Tissue

Procedures for immunofluorescence histochemical staining have previously been described. Briefly, tumor specimens were deparaffinized, rehydrated, and antigen retrieved as recommend by the manufacturer. To reduce autofluorescence, each specimen was incubated for 30 minutes in 0.1% sodium borohydride in PBS at room temperature and then was incubated for 5 minutes in 0.5% Sudan black in 70% ethanol. After washing in distilled water, each sample was incubated for 10 minutes at room temperature with a blocking solution of PBS plus 1% bovine serum albumin and 2% fetal calf serum before incubation with the primary antibody. The specimens were then labeled with a cocktail of primary antibodies overnight at 4°C, unbounded primary antibodies were washed away, and the specimens were labeled with Alexa Fluor–conjugated secondary antibodies at room temperature for another 1 hour. Hoechst 33342 dye was used to stain tissue nuclei. To control for nonspecific binding of fluorochrome-conjugated antibodies, sections were subjected to the same staining procedures except that the primary antibody was omitted.

Animals

Six- to 8-week-old female BALB/c nude mice (BioLASCO, Taipei, Taiwan) were maintained on a standard diet of chow and water ad libitum. All the procedures and protocols were approved by the Institutional Animal Care and Use Committee of Chang Gung Memorial Hospital.

Fluorescent Labeling of Cancer Cells

Cells (1 × 10⁷ cells/mL) were incubated for 30 minutes at 37°C in Opti-MEM (a reduced-serum medium) containing the fluorescent dye Green CMFDA or Orange CMRA (10 μmol/L) as described elsewhere. After washing, the cells were incubated for an additional 30 minutes with dye-free PBS, washed, and harvested for injection into nude mice.

Locomotion of Tumor Cells to Lungs of Nude Mice

To measure lung metastasis as described elsewhere, stable radioresistant and wild-type CaSki cells labeled with red or green fluorescent dye were mixed at a ratio of 1:1. A total of 4 × 10⁶ cells were injected through the tail vein into each mouse. Lungs were harvested 1 or 24 hours after injection, and lung tissue was sectioned at 10 μm in a cryostat to observe the locomotion of tumor cells to lungs. The ratio of green to red fluorescent cells in the injected suspension was measured by fluorescence microscopy.
Statistical Analysis

The data are expressed as mean ± SD and were analyzed by one-way analysis of variance using Prism software version 4.02 (GraphPad Software Inc., La Jolla, CA). The Tukey test was used to assess differences between individual treatment groups, and the Dunnett test was used to compare treatment groups versus controls (once significance was established) using the F test. The log-rank test was used to assess differences between groups of Kaplan-Meier survival curves. Student’s t-test was used to compare two samples.

Results

Cell Migration and Wound Healing Are Increased in Radioresistant C33A and CaSki Cells but Not in HeLa Cells

First, to further confirm that these surviving populations after 6-Gy irradiation treatments selected as stable radioresistant clones among HeLa, CaSki, and C33A cells were really radioresistant, we performed the clonogenic survival curve assay to examine their radiosensitivity again. The survival curve showed the reduced radiosensitivity of the stable radioresistant clones of HeLa, CaSki, and C33A cells to irradiation compared with their parental wild-type cells (Figure 1A). Furthermore, the wound-healing assay and time-lapse recording of cell migration were performed on the radioresistant CaSki, C33A, and HeLa cells, which had undergone stable selection for 1 month after irradiation. The wound-healing rate of wild-type control CaSki cells was significantly faster than that of wild-type HeLa or C33A cells (Figure 1B). The radioresistant C33A and CaSki cells showed a significantly increased healing rate compared with their wild-type cells. This was not the case for the radioresistant HeLa cells, however (Figure 1B and C). This phenomenon was consistent and reproducible among the various clones of the three cell lines. To further confirm that radioresistance was associated with increased cell migration, time-lapse recordings of C33A, CaSki, and HeLa cell movement were made hourly to trace the paths of migrating cells. Consistently, the radioresistant C33A and CaSki cells migrated faster than their wild-type counterparts. In contrast, no such difference was observed for HeLa cells (Figure 1D and E).

Increase in Cell Migration Persists in Radioresistant C33A and CaSki Cells Cultured for Up to 6 Months

To assess whether the faster migration rate of the radioresistant C33A and CaSki cells was a stable phenotype, radioresistant C33A, CaSki, and HeLa cells were cultured for 1, 3, and 6 months after a sublethal 6-Gy dose of irradiation and then were subjected to time-lapse record-

Figure 1. Radioresistant (RR) C33A and CaSki cells exhibit enhanced migration in vitro. A: Radiation survival curves were performed on cells in log phase growth. Wild-type (WT) or RR cells were plated, irradiated, and assayed for clonogenic survival at the doses indicated (as described in Materials and Methods). All clonogenic assays were repeated at least three times. Shown are mean ± SD values. Solid and dotted curves represent WT and RR cells, respectively. B: Representative phase-contrast micrographs depicting scratched monolayers of serum-starved WT and RR HeLa, C33A, and CaSki cells at 0 and 8 hours. Scale bar = 100 μm. The results are from various independent clones of HeLa, C33A, and CaSki, each clone was repeated at least three times, and the results were similar. C: Summary of wound healing 8 hours after scratching. Mean ± SD values of various independent clones of HeLa, C33A, and CaSki cells are shown. D: Representative cell migration trace from a time-lapse recording of cell migration over 8 hours. The results are representative of various independent clones of HeLa, C33A, and CaSki cells, and each clone was repeated at least three times. Scale bar = 100 μm. E: Summary of migration speed from a time-lapse recording of cell migration over 8 hours. Mean ± SD values of various independent clones are shown. F: Summary of time-lapse recording data for the various clones of the three WT and RR cell lines. Data are the mean ± SD of three independent experiments. The cells underwent stable selection for 1, 5, and 6 months after irradiation, and at least 20 cells per individual sample were counted. *P < 0.05, **P < 0.01.
We found that radioresistant C33A and CaSki cells had persistently higher cell migration than did their wild-type cells (Figure 1F). In contrast, the migration rate of radioresistant HeLa cells did not differ from that of wild-type cells at any time points (Figure 1F). These data suggest that the increased cell migration after irradiation was consistently associated with radioresistance in C33A and CaSki cells but not in HeLa cells.

Higher Potential for Lung Metastasis of the Radioresistant CaSki Cells in Nude Mice

To investigate whether radioresistance-enhanced cervical cancer cell migration causes potential metastasis to lung in vivo, we injected wild-type and radioresistant CaSki cells, which had higher cell mobility, into BALB/c nude mice via the tail vein. To track injected cells, CaSki cells were labeled with fluorescent dyes before injection (wild-type CaSki cells were labeled green and radioresistant CaSki cells were labeled red). Labeled cells were mixed in a 1:1 ratio and were injected into the tail vein. Mice were sacrificed 1 or 24 hours after injection, and the harvested lungs were then used to produce frozen sections. One hour after injection, the ratio of radioresistant CaSki cells (red) to wild-type cells (green) was nearly 1:1 (after normalizing for the composition of the suspension before injection), indicating that the injected cells metastasized equivalently to the lung at an early initiation time (Figure 2A). After 24 hours, however, the mean ± SD ratio of radioresistant CaSki cells to wild-type cells had increased dramatically: 1.67 ± 0.17 at 24 hours compared with 0.96 ± 0.05 at 1 hour after injection ($P < 0.05$) (Figure 2B; quantified in Figure 2C). To exclude potential effects of dye-labeling efficiency, we swapped the two dyes used to label the radioresistant CaSki and wild-type cells. The results (Figure 2D) were consistent with those obtained with the first dye-cell combinations. Furthermore, the long-term survival rate was also evaluated 70 days after tail vein injection of wild-type or radioresistant CaSki cells in vivo. Figure 2E shows a significantly lower survival rate in mice that received radioresistant CaSki cells compared with mice that received wild-type CaSki cells.

Increased Cell Migration of Radioresistant C33A and CaSki Cells Is Mediated by K-Ras

To explore whether the radioresistance-enhanced C33A and CaSki cell migration was mediated by Ras activation, we first established which Ras protein was affected by irradiation. As shown in Figure 3A, H-Ras was ubiquitously expressed in all of the wild-type and radioresistant cells. In contrast, K-Ras expression was dramatically increased in radioresistant C33A and CaSki cells compared with in wild-type cells, but this was not the case for HeLa cells (Figure 3A and see Supplemental Figure S1 at http://ajp.amjpathol.org). In addition, the level of c-Raf, phosphorylated at Ser338 (ie, activated), was dramatically increased in radioresistant C33A and CaSki cells compared with in wild-type cells, whereas there was no such difference in HeLa cells. This phenomenon was consistent among all the clones of the three cell lines (Figure 3A and see Supplemental Figure S2 at http://ajp.amjpathol.org). Thus, the increased level of activated c-Raf in radioresistant C33A and CaSki cells, presumably a consequence of activation by K-Ras, correlated with their increased migration phenotype.
We next aimed to test whether increased in c-Raf phosphorylation could be due to activating mutations of K-Ras after irradiation. Genomic DNA isolated from parental and radioresistant clones were subjected to sequencing to screen for any potential activating mutations in all six exons of K-Ras (performed by the Genomic Center of National Cheng Kung University, Tainan, Taiwan). As shown in Supplemental Figure S3 (available at http://ajp.amipathol.org), there was no activating mutation of K-Ras in either parental or radioresistant cell clones. These results suggest that increased c-Raf phosphorylation in CaSki and C33A cells is mediated by up-regulation of K-Ras.

We next performed RNA interference studies entailing specific siRNA-mediated knockdown of K-Ras. Transfection of CaSki cells with K-Ras siRNA abolished radioresistance-enhanced K-Ras expression (Figure 3B), thereby inhibiting the downstream phosphorylation of c-Raf (at Ser338). A similar knockdown of endogenous H-Ras caused only a slight inhibition of c-Raf phosphorylation (Figure 3B). Knockdown of endogenous K-Ras did not affect the basal migration rate of wild-type CaSki cells.

Figure 3. Increased migration of radioresistant C33A and CaSki cells is mediated by K-Ras. Western blots and time-lapse recordings of serum-starved wild-type (WT) and radioresistant (RR) C33A, CaSki, and HeLa cells that had been cultured to confluence. A: Representative Western blot showing the levels of K-Ras, H-Ras, c-Raf, and phosphorylated c-Raf in WT and RR HeLa, C33A, and CaSki cells. β-actin served as internal control. Three independent experiments were performed. B: Levels of K-Ras, H-Ras, c-Raf, and phosphorylated c-Raf in WT and RR CaSki cells transiently transfected with a specific siRNA against H-Ras (H-Rasi) or K-Ras (K-Rasi) for 72 hours. β-actin served as internal control. The results are representative of three experiments. C: Time-lapse recording results of cell migration of WT and RR HeLa, CaSki, and C33A cells after specific knockdown of K-Ras or H-Ras. At least 20 cells per individual were counted. Data are the mean ± SD of three independent experiments. *P < 0.05, **P < 0.01. scr, scrambled/negative control siRNA.

Figure 4. Involvement of c-Raf phosphorylation in the radioresistance-associated enhanced migration of C33A and CaSki cells. A: Representative image showing specific knockdown of c-Raf by siRNA constructs (c-Rafi) for 72 hours from three independent experiments. β-actin served as internal control. c-Rafi #1, siRNA of c-Raf duplex1; c-Rafi #2, siRNA of c-Raf duplex2; c-Rafi #3, siRNA of c-Raf duplex3. B: Summary of time-lapse recordings of the migration of radioresistant (RR) C33A and CaSki cells from three independent experiments. At least 20 cells per individual were counted. C: Representative images showing phosphorylated c-Raf expression (green fluorescence) in wild-type (WT) and RR CaSki cells. White arrows indicate phosphorylated c-Raf that accumulated at the leading edge during wound closure. Specific siRNA-mediated knockdown of c-Raf decreased c-Raf expression and wound healing. Black arrows indicate the migrating leading edge at 8 hours. Scale bar = 100 μm. Left panels show control images. Three independent experiments were performed, and the results were similar. D: Summary of wound healing 8 hours after scratching of CaSki cells. Data are the mean ± SD of three independent experiments. *P < 0.05, **P < 0.01. scr, scrambled/negative control siRNA.
but significantly inhibited the increased cell migration exhibited by radioresistant CaSki cells (Figure 3C). In contrast, knockdown of endogenous H-Ras significantly reduced the basal migration rate for wild-type and radioresistant CaSki cells. A similar phenomenon was observed for radioresistant C33A cells (Figure 3C). This phenomenon was also consistently observed among all the various clones of the three cell lines (see Supplemental Figure S2 at http://ajp.amjpathol.org). These results demonstrate that H-Ras is involved in determining the basal rate of cell migration, but K-Ras plays a key role in mediating the radioresistance-enhanced migration of C33A and CaSki cells, possibly via c-Raf activation.

**Phosphorylation of c-Raf Plays a Critical Role in the Increased Cell Migration of Radioresistant C33A and CaSki Cells**

To further explore the involvement of c-Raf in increased cell migration of radioresistant C33A and CaSki cells, c-Raf-specific siRNAs were used to knock down c-Raf expression. As shown in Figure 4A, 72 hours after transfection, all three specific siRNAs knocked down the levels of total c-Raf and phosphorylated c-Raf. K-Ras levels increased slightly after c-Raf knockdown, which may be due to compensatory feedback for Ras expression. The specific siRNA-mediated knockdown of c-Raf also attenuated the radioresistance-enhanced migration of C33A and CaSki cells (Figure 4B). In the wound-healing assay (Figure 4C), radioresistant CaSki cells migrated faster than did wild-type cells. The increased CaSki cell migration was further demonstrated by immunofluorescence staining of phosphorylated c-Raf (at Ser338). Strong expression was observed in the leading edge (shown as white arrows in Figure 4C) of radioresistant CaSki cells compared with wild-type cells. Transfection with a specific c-Raf siRNA abolished c-Raf expression and attenuated the radioresistance-enhanced wound-healing rate of CaSki cells (Figure 4D). These data show that phosphorylation of c-Raf is critical for the K-Ras–mediated increase in cell migration of radioresistant C33A and CaSki cells.

**p38, but Not ERK or JNK, Is the Effector for the K-Ras–Mediated Increase in Migration of Radioresistant C33A and CaSki Cells**

It is well established that p38, JNK, and ERK are downstream effectors of c-Raf. We, therefore, explored the phosphorylation and, hence, activation of these proteins to determine which effectors in the K-Ras/c-Raf signaling cascade are involved in the increased migration of radioresistant C33A and CaSki cells. As shown in Figure 5A, increased p38 phosphorylation (but not ERK1/2 or JNK phosphorylation) was evident in both the radioresistant C33A and CaSki cells. To determine whether p38 activation is involved in the K-Ras/c-Raf–mediated enhancement of C33A or CaSki cell migration, we transfected an siRNA specific for H-Ras, K-Ras, or c-Raf into cells and expressed each siRNA for 72 hours. p38 phosphorylation was significantly reduced on knockdown of c-Raf or K-Ras but not of H-Ras (Figure 5B). We next used a selective inhibitor of p38, MAPK/ERK, JNK, and vehicle control (dimethyl sulfoxide) to investigate the effect of inhibiting the activation of these intracellular signaling mediators on the radioresistance-enhanced migration of C33A and CaSki cells. In the wound-healing assay (Figure 5C), the increased migration of radioresistant C33A and CaSki cells was significantly inhibited by the p38 inhibitor (SB203580), but not the ERK (U0126) or JNK (SP600125) inhibitor, significantly inhibited the migration of RR C33A and CaSki cells. The results are the mean ± SD of three independent experiments. *P < 0.05, **P < 0.01. scr, scrambled/negative control siRNA.

**Increased K-Ras Expression in Locally Recurring Cervical Cancer Tumors and in Distant Metastases after Radiotherapy**

Finally, we used immunofluorescence histochemical staining to examine K-Ras expression in specimens collected from three paired patients in primary cervical cancer tumors before radiotherapy and locally recurring cervical tumors and distant metastases collected after radiotherapy. Table 1 lists the patient information, including the date that the patients received radiotherapy, the
time of local recurrent sample isolation, the time of distal metastatic sample isolation, and the distal metastatic sites. Because EGFR is overexpressed in a variety of cancers, we used a primary rabbit monoclonal antibody against EGFR to identify tumor areas (green fluorescence) in each individual specimen. K-Ras–positive cells were identified by red fluorescence, and cell nuclei were counterstained with Hoechst 33342 (blue fluorescence). As shown in Figure 6, the cervical cancer tissues collected before radiotherapy showed weak K-Ras immunoreactivity. In contrast, the radioresistant tumor tissues collected after radiotherapy showed obviously increased K-Ras immunoreactivity, especially in the EGFR-positive tumor sites. Importantly, the strong K-Ras staining was seen not only in the locally recurring cervical tumors but also in tissues in distant metastases, such as lung, small bowel, and mediastinal lymphoid nodes. Similar phenomena were also observed in specimens from another four paired patients (see Supplemental Figure S4 at http://ajp.amjpathol.org). A similar pattern of phosphorylation of c-Raf and p38 was also found in locally recurrent radioresistant tumors and distant metastases (data not shown). Taken together, these results suggest that the K-Ras/c-Raf/p38 signaling cascade might be responsible, at least in part, for the observed radioresistance-enhanced cervical tumor metastasis.

Discussion

In this study, we established three stable radioresistant human cervical cancer cell lines and investigated the biochemical pathways involved in radioresistance-enhanced cell migration. These data demonstrate that a sublethal dose of radiation promotes cervical cancer metastatic potential in CaSki and C33A cells. This finding is consistent with the clinical observation that failure of radiotherapy in cervical cancers is usually associated with metastasis. We also showed that radioresistance-enhanced cell migration was mediated by the K-Ras/c-Raf/p38 signaling pathway. Clinical examination confirmed activation of the K-Ras/c-Raf/p38 pathway in locally recurrent and distant metastatic tumors but not appreciably in primary tumors. These results suggest that the use of inhibitors that block K-Ras/c-Raf/p38 signaling combined with radiotherapy may reduce the risk of generating radioresistance-associated metastases of cervical cancers.

Migration and invasion are important prerequisites for cancer progression and metastasis. The fact that radiation may enhance the invasive and metastatic potential of cancer cells suggests that the classic radiotherapy approach to treating cervical cancer may require an additional component: an antimetastasis drug regimen. A radiation-induced increase in invasive potential has been shown in human pancreatic cancer cells, and a sublethal dose of radiation can enhance the invasiveness of hepatocellular carcinomas and neurogenic tumors. In a mouse model, radiotherapy for a primary Lewis lung carcinoma accelerated metastasis. These findings support the present results that radioresistance, after a sublethal dose of radiation, is associated with enhanced cell migration and metastatic potential. However, the mecha-

Table 1. Patient Information Used in This Study

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<th>Patient no.</th>
<th>Radiotherapy date</th>
<th>Local recurrent sample isolation date</th>
<th>Distal metastatic sample isolation date</th>
<th>Metastatic sites</th>
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Figure 6. Immunofluorescence histochemical staining of K-Ras in human cervical cancer tissue specimens before and after radiotherapy in locally recurring tumors and distant metastases to multiple organs from three patients. Images showing formalin-fixed, paraffin-embedded tissues were sectioned and stained with anti-K-Ras (red fluorescence) or anti-EGFR (green fluorescence). Yellow fluorescence indicates co-localization of K-Ras and EGFR in the merged images. Hoechst 33342 staining is shown in blue. Scale bars: 100 μm. The results are from three patients (patients 1 to 3 in Table 1) with human cervical cancer who collected the individual complete set of tissue specimens, including before and after radiotherapy in locally recurring tumors and distant metastases.
nism underlying radioresistance-enhanced cell invasive-
ess differs between cancer cells, and prevention of
radiation-induced cancer cell metastasis requires con-
sideration of the cancer type. Based on the present stud-
ies, combined radiotherapy and a selective inhibitor of
K-Ras/c-Raf/p38 signaling may eliminate the metastatic potential of cervical cancers.

Ras signaling pathways are integral to cancer cell prolif-
eration and radioresistance. Recent evidence has
shown that Ras activation plays a critical role in cell
mobility: ectopic expression of K-Ras (V12) down-regu-
lated the activity of Rac1 and RhoA, resulting in reduced
levels of subcortical actin filaments and stress fibers,
which might contribute to human pancreatic tumor cell
migration and invasion. On activation by H-Ras, the
ERK/MAPK and AKT pathways promoted the invasiv-
eness of MCF10A human mammary epithelial cells. H-
Ras has also been found to promote invasion and me-
tastasis via a leukotriene receptor, BLT2, which links to a
signaling cascade involving NF-κB stimulation in Rat2-
HO6 cells. In contrast to the ubiquitous expression of
H-Ras in wild-type and radioresistant CaSkii or C33A
cells, K-Ras levels were low in wild-type CaSkii and
C33A cells but dramatically elevated in their radiores-
sistant cells. These data further demonstrate that in
cervical cancer cells, oncogenic K-Ras plays a prom-
inent metastatic role in radioresistance-enhanced
migration.

c-Raf is one of the best-characterized Ras effector
proteins. These data show that K-Ras knockdown re-
sulted in a near-complete block of c-Raf phosphoryla-
tion on Ser338, whereas H-Ras knockdown had relatively little
effect in this regard. The attenuated phosphorylation of
c-Raf greatly diminished cell migration. Recent studies
have also demonstrated the distinct ability of different
Ras isoforms to activate c-Raf. For example, compared with
H-Ras, K-Ras is a more potent activator of c-Raf in
BH4 and COS cells, and K-Ras activates c-Raf more
efficiently than H-Ras or N-Ras in COS cells. These
findings support the result that K-Ras, but not
H-Ras, is a potent activator of c-Raf in radioresistance-
enhanced migration of CaSkii and C33A cells. Recent
studies have confirmed that expression of oncogenic Ras
does not necessarily correlate with increased MEK/ERK
activity; several human ductal pancreatic carcinoma cell
lines, colon carcinoma cell lines, and astrocytoma
cells express constitutively active K-Ras but do not
exhibit constitutively active ERK. We herein also identified a
MEK/ERK-independent effector pathway involved in the
K-Ras–mediated enhancement of cell migration in ra-
dioresistant cervical cancer cells via c-Raf/p38. The
involvement of p38 in cell migration has been shown for
various cell types, and putative substrates of p38-
mediated response involve MAPK 2 and 3, heat shock
protein, and paxillin. In MCF10A breast epithelial cells, p38 activation is mediated by H-Ras and Rac1-
MAPK kinase 3/6 interaction. However, the underlying
mechanistic link to cell mobility is not fully understood.

In conclusion, we showed that a sublethal dose of
radiation enhances the metastatic potential of human cer-
vical cancer cells and that this phenomenon is mediated
by K-Ras/c-Raf/p38 signaling. The clinical implication of
this study is the critical need to suppress the radiation-
enhanced metastatic potential of human cervical cancer
cells. With further clarification of the signal transduction
cascades involved in this process, inhibition of the K-Ras/
c-Raf/p38 signaling pathway may provide an additional
targeted approach to modulate metastatic signaling,
thereby retaining the therapeutic benefit of radiotherapy
for cervical cancers.

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