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Pim-1 Kinase Expression Predicts Radiation Response in Squamocellular Carcinoma of Head and Neck and Is under the Control of Epidermal Growth Factor Receptor[1,2]

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Abstract

Pim-1 is an oncogenic serine/threonine kinase with poorly defined function in epithelial cancers. In this study, we determined 1) associations of Pim-1 expression with clinicopathological parameters including responsiveness to irradiation in squamocellular cancers of head and neck and 2) how Pim-1 expression is controlled subsequent to irradiation. Moderate to high expression of Pim-1 correlated to poor response to radiation therapy ($P = .003$). It is also associated to the expression of epidermal growth factor receptor (EGFR, $P < .0001$), which has been shown to be activated by irradiation. In radioresistant tumors, irradiation promoted nuclear translocation of Pim-1 ($P < .005$). When directly testing EGFR dependence of Pim-1 expression, up-regulation and nuclear translocation of Pim-1 could be induced through stimulation of EGFR with its ligands EGF or transforming growth factor α. Both ligand- and irradiation-induced changes in Pim-1 expression and localization could be inhibited by the monoclonal anti-EGFR antibody cetuximab and by the tyrosine kinase inhibitor gefitinib also targeting EGFR. These results suggest that irradiation-induced activation of EGFR upregulates Pim-1, and Pim-1 may be used as a novel predictive marker of radiation response in patients with squamocellular cancers of head and neck.

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Introduction

The *pim-1* gene encodes a serine/threonine kinase that has been implicated in cytokine receptor–initiated signaling as well as in cellular survival [1,2]. It has also been shown to regulate activities of several transcription factors and cell cycle regulators [3–8].

The *pim-1* gene was originally identified as a proviral integration site for the Moloney murine leukemia virus [9] and was shown to function as a proto-oncogene. Alone, *pim-1* is only weakly oncogenic, but a series of studies with transgenic mouse models has demonstrated that *pim-1* can efficiently cooperate with other oncogenes such as *myc* family

Abbreviations: EGFR, epidermal growth factor receptor; HNSCC, squamocellular cancers of head and neck; TGF-α, transforming growth factor α

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members, *bcl-2* or *gfi-1* in the development of B- or T-cell lymphomas [10–13]. Elevated levels of *pim-1* have also been found in human lymphoid and myeloid malignancies [14]. Although recent studies have identified *pim-1* as a prognostic marker for one epithelial cancer type, prostate cancer [15], its behavior during malignant transformation of epithelial cells and its correlation to clinicopathological parameters have remained less well known. Therefore, the purpose of our study was to analyze the expression of the Pim-1 kinase in squamocellular cancers of head and neck (HNSCC) to establish a possible association among the expression status, clinical behavior, and treatment response of the tumors. The strong association observed between Pim-1 and epidermal growth factor receptor (EGFR) expression led to further analyses on regulation of Pim-1 by EGFR triggering.

Materials and Methods

Patients

Seventy-one patients with histopathologically defined HNSCC treated at the Department of Oncology and Radiotherapy and at the Department of Otorhinolaryngology – Head and Neck Surgery at Turku University Hospital were included in the study. The median age of the study patients was 66 years (ranged from 25 to 97 years) at the time of diagnosis, representing that of the general population with HNSCC. The patient characteristics are listed in Table W1, and their treatments are listed in Table W2. After the follow-up period of 60 months, 23 patients were alive. Twenty-seven died of HNSCC and 17 died from other reasons. In three cases, the cause of death was not known, and one patient died of a cancer treatment. Primary diagnostic biopsy samples were taken from all 71 primary tumors before any therapy was given. In addition, surgical samples were taken after preoperative irradiation from all patients undergoing surgery (n = 52). The expressions of Pim-1 and EGFR were analyzed in those surgical samples, which were Pim-1–positive in the primary diagnostic samples before irradiation.

All tissue sampling and experimental procedures were approved by the institutional human research committee, and informed consent of the patients was obtained.

Primary Antibodies

Mouse monoclonal anti–Pim-1 antibody (19F7) raised against a C-terminal peptide was a kind gift from Dr. Michael Lilly (Loma Linda University, Loma Linda, CA). This antibody specifically recognizes Pim-1 but not the other Pim family members. Anti-EGFR (clone 25) antibody was from Novocastra Laboratories, Ltd. (Newcastle upon Tyne, United Kingdom) and the monoclonal antibody 3G6 (against chicken T cells) used as a negative control was made in house.

Histologic Diagnosis and Immunoperoxidase Staining

At the time of diagnosis, histopathological evaluation revealed that all tumors displayed typical features of squamous cell carcinoma. After preoperative radiotherapy, tumors were considered to have viable tumor tissue, if an experienced pathologist observed any well-preserved tumor tissue histology with mitotic figures. If viable tumor tissue was observed after preoperative radiotherapy, the tumor was scored as a nonresponder to irradiation.

The expression level and distribution of Pim-1 and EGFR proteins were determined using formalin-fixed paraffin-embedded sections and indirect immunoperoxidase staining method as described [16]. Briefly, sections were stained with primary antibodies followed by reagents of the Vectastain Elite ABC kit (Vector Laboratories, Inc., Peterborough, United Kingdom). 3,3-Diaminobenzidine in Tris-buffered saline containing 0.03% H_2O_2 was used as the substrate for the peroxidase-mediated reaction.

Antibody staining was scored by using a semiquantitative scale combining both intensity and percentage of positive cells. The interexperimental variation in staining intensity was controlled by adjusting the scoring to the intensity of Pim-1 staining in normal tumor-infiltrating lymphocytes present in variable numbers in each tumor. Score 0 (negative) represents practically no Pim-1 or EGFR positivity, whereas score 3 (strong) was given to samples in which practically all tumor cells were strongly positive. Score 1 (weak) was given to samples expressing weakly positive cells comparable to the level of Pim-1 in normal keratinocytes, and score 2 (moderate) was adjusted to cover the staining patterns in between 1 and 3 (see also examples in Figure 1). Scoring was performed by two independent readers. The borderline cases (n = 6) were jointly reviewed, and a consensus was sought.

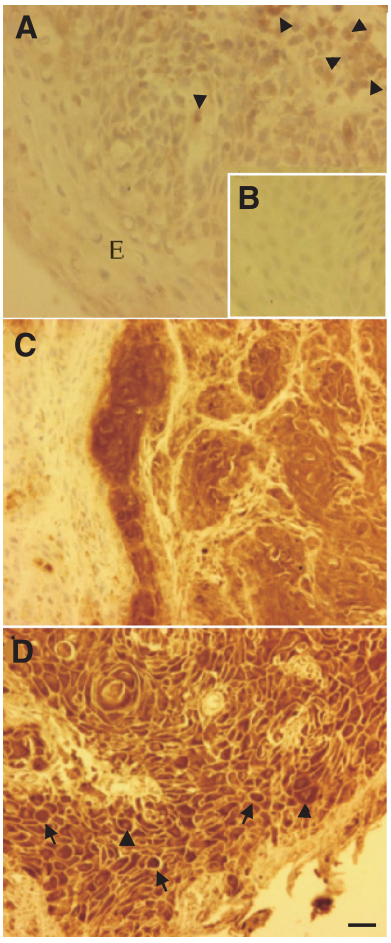

Figure 1. Pim-1 protein is overexpressed in a subset of HNSCCs. (A) Tonsil tissue stained with anti–Pim-1 antibody exhibiting weak expression of Pim-1 in normal epithelium (E). Some normal lymphocytes expressing Pim-1 are pointed out by arrowheads. (B) Staining of the same sample as in (A) with a negative control antibody. (C) Moderate expression of Pim-1 in grade 1 carcinoma. (D) Strong expression of Pim-1 in grade 1 carcinoma. The longer arrows point to cells with nuclear Pim-1 expression, and the arrowheads point to cells with prominent cytoplasmic Pim-1 localization. Bar, 50 μm.

Cells and Cell Lines

HaCaT, a spontaneously transformed human adult epithelial cell line with full epidermal differentiation capacity, was a kind gift from Professor N.E. Fusenig (German Cancer Research Centre, Heidelberg, Germany) [17]. The HNSCC cell lines were established from patients with cancer of the head and neck region as previously described [18].

Tumor Model

To determine the usability of HNSCC lines in our studies, tumor nodules were grown in severe combined immunodeficient (SCID) mice, and their Pim-1 expression was determined. HaCaT cells and five human HNSCC cell lines were injected subcutaneously into the back of SCID mice. During the following 25 to 41 days, the tumor nodules became clearly visible, and the animals were killed. The nodules were excised, frozen in liquid nitrogen, and prepared for immunofluorescence staining. The permission for animal experiments was obtained from the Ethical Committee of Turku University.

Growth Factors and Inhibitors

Cultured HNSCC cell lines were stimulated with or without 20 and 50 ng/ml EGFR ligands, EGF (Intergen, Edinburgh, United Kingdom) or transforming growth factor α (TGF-α; R&D, Oxon, United Kingdom) for 18 or 72 hours. To inhibit EGFR signaling, anti-EGFR antibody cetuximab (10 nM; Merck, Espoo, Finland) or EGFR targeting tyrosine kinase inhibitor gefitinib (10 μM; AstraZeneca, Espoo, Finland) were added together with EGF for 72 hours. A tumor-promoting agent (TPA), 12-*O*-tetradecanoyl-phorbol-13-acetate (15 ng/ml; Sigma-Aldrich, St. Louis, MO), was used as a control. In the irradiation experiments, cetuximab and gefitinib were administered 24 hours before the irradiation (2 Gy), and the stainings for Pim-1 expression were performed 48 hours after irradiation.

Small Interfering RNA Treatments, Viability Determinations, and Western Blot Analysis

Monolayers of HNSCC cells were transfected with Oligofectamine (Invitrogen, Carlsbad, CA) according to manufacturer's instructions using Dharmacon (Chicago, IL) ON-TARGET plus small interfering RNA (siRNA) oligonucleotides that were either nontargeting (NT) or specifically targeted Pim-1 siRNA. Twenty-four hours later, the cells were irradiated (2 or 3 Gy) and incubated for an additional 48 hours before the analysis of cell viability. Viability was determined by trypan blue staining. Ten microscopic fields (magnification, ×400) of each well were counted for trypan blue–positive cells. In addition, cell death was determined using flow cytometry. Shortly, tumor cells were collected in PBS and then treated with hypotonic buffer (1% Triton X-100, 0.05 mg/ml propidium iodide in PBS) for 20 minutes at 4°C. DNA content was measured using FACSCalibur flow cytometer with CellQuest Pro software (Becton Dickinson, CA). Apoptosis analysis was performed using ModFit LT (Verity Software House, Inc., Topsham, ME) cell cycle modeling software by calculating the subdiploid population.

Silencing of Pim-1 expression was confirmed by Western blot analysis and immunofluorescence (as explained in the next section). For Western blot analysis, the cells were lysed, and protein concentrations were measured. Equal amounts of protein from each sample were separated by SDS-PAGE, blotted onto polyvinylidene difluoride membrane and analyzed for Pim-1 expression using anti–Pim-1 antibody (sc-13513, clone 12H8; Santa Cruz, Heidelberg, Germany) followed by ECL Plus reagents (GE Healthcare, Helsinki, Finland) and autoradiography.

Immunofluorescence Staining

HaCaT and HNSCC cell lines were grown on coverslips. These cells were fixed with 4% paraformaldehyde for 20 minutes, treated with 0.2% Nonidet P-40 for 5 minutes and subjected to indirect immunofluorescence staining as described [16] with anti–Pim-1, anti-EGFR, or the negative control antibody, 3G6 against chicken T cells. Fluorescein isothiocyanate–conjugated anti–mouse immunoglobulin G (Sigma) containing 5% of normal human serum was used as a secondary antibody. The stainings were analyzed using Olympus 60BX microscope (Hamburg, Germany). Subcellular localization and Pim-1 positivity were scored by two independent readers. The intensity scores were as follows: +, weak; ++, moderate; +++, strong.

Statistical Analyses

Statistical analyses were performed with the SAS Enterprise Guide 2 computer program for the personal computer (SAS Institute, Inc., Espoo, Finland). To test the associations between different biological variables and between the Pim-1 expression and clinicopathological parameters, χ^2 test was used for trends, the Fisher exact test was used for categorical variables, and Pearson correlation coefficient test was used for continuous data. Cumulative survival between the groups for each variable studied was compared with the log-rank and Wilcoxon tests. McNemar test was used to analyze the changes in nuclear localization after radiation therapy.

Results

Pim-1 Expression in HNSCC

Pim-1 protein expression was analyzed by immunoperoxidase staining in squamocellular epithelium of normal tonsils and in 71 primary diagnostic biopsy samples of HNSCC (Tables W1 and W2 for patient characteristics). Pim-1 expression was weak or negative in keratinocytes of normal tonsil tissues, whereas many of tonsillar lymphocytes expressed clearly detectable levels of Pim-1 (Figure 1*A*). Of 71 HNSCCs, 29 (41%) showed weak or negative Pim-1 expression that was comparable to Pim-1 expression detected in keratinocytes of normal tonsil tissue. However, 32 (45%) of 71 HNSCCs expressed moderate levels of Pim-1, and a strong Pim-1 expression was detected in 10 (14%) of 71 HNSCCs. Thus, the majority (59%) of HNSCC overexpressed Pim-1 when compared with the expression level in normal keratinocytes. In Pim-1–expressing tumors, the intracellular localization of Pim-1 was heterogenous. Although many cells exhibited nuclear localization of Pim-1, also cells with cytoplasmic Pim-1 expression were found in each Pim-1–positive tumor (Figure 1, *C* and *D*).

Association of Pim-1 Expression with Clinicopathological Parameters

Pim-1 expression in primary diagnostic biopsy samples did not correlate with the histological grade of differentiation, sex, age at diagnosis, tumor size, or presence of lymph node metastases (data not shown). However, Pim-1 expression showed a trend of association with overall survival ($P = .09$; Figure 2*A*). Tumor size and nodal status were significantly associated with survival ($P < .05$; data not shown). These findings suggest that, in HNSCC, relatively high Pim-1 expression levels tend to associate with poor prognosis.

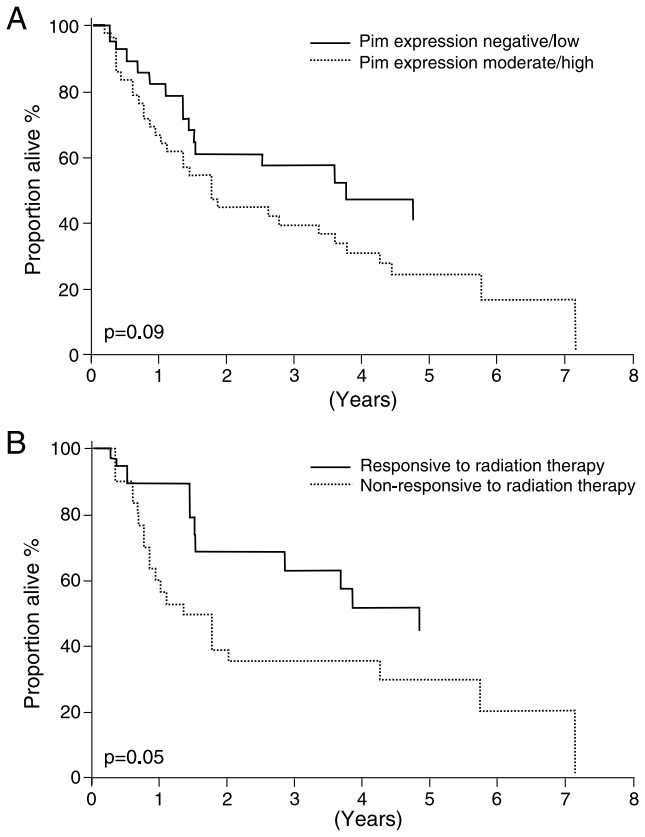

Figure 2. Patients with tumors expressing high levels of Pim-1 tend to have a poor prognosis, whereas patients with tumors responding to radiation survive longer. Kaplan-Meier survival curves of patients categorized according to Pim-1 expression (A) and radiation response (B) of their tumors.

Pim-1 Expression Is Associated with Poor Response to Irradiation and Expression of EGFR

The standard treatment regimen for HNSCC in Turku University Hospital includes initial preoperative radiotherapy followed by surgery 3 weeks later. This provided us with an opportunity to study cellular and molecular responses to irradiation in the tumor samples removed in the operation after irradiation. Sixty-four of the HNSCCs examined were initially subjected to radiotherapy and 52 of them were operated on. Viable tumor tissue was observed in histological analyses of operated tumor samples more frequently in tumors with moderate to strong Pim-1 expression than in tumors with low or no Pim-1 expression (P = .003). These findings indicate that upregulated expression of Pim-1 associates with poor response to irradiation in HNSCC (Table 1).

Expression of EGFR has previously been linked to radiation resistance in HNSCC [19]. As Pim-1 expression behaved similarly as has been described for EGFR, we analyzed EGFR expression also in our series. Sixty-one primary diagnostic biopsy samples and 17 Pim-1–positive postoperative samples were available for immunohistochemical stainings. One of the primary diagnostic biopsy samples was negative, 26 expressed low levels, 27 moderate and 7 high levels of EGFR. As expected, expression levels of EGFR inversely correlated with responsiveness to irradiation (P = .018; Table 1). Furthermore, EGFR expression strongly associated with Pim-1 expression both in primary diagnostic biopsy samples and in surgical samples taken after irradiation (P < .0001). In contrast to Pim-1 and EGFR expression, the classic prognostic parameters such as tumor size, nodal status, and histological grade did not significantly associate with the radiation response (Table 1). However, favorable response to radiation therapy was significantly associated with survival (P = .05; Figure 2*B*). These data indicate that both Pim-1 and EGFR expression are markers of poor response to irradiation in HNSCC.

Tumors with Poor Response to Irradiation Demonstrate Nuclear Localization of Pim-1

Irradiation has been reported to change intracellular localization of EGFR in radioresistant tumor cells, and it also causes autophosphorylation and activation of EGFR [19,20]. Because nuclear localization has recently been shown to be required for oncogenicity of Pim-1 in Burkitt lymphoma [21], we wanted to analyze the effects of irradiation on subcellular localization of Pim-1. Therefore, Pim-1 protein expression was analyzed from 17 surgical samples that included viable radioresistant tumor tissue after preoperative irradiation. Only patients who had Pim-1–positive tumor cells in the primary diagnostic biopsy samples were included in the analysis. Irradiation did not induce any obvious changes in subcellular localization of Pim-1 in any of those tumors (9/17), which had prominent nuclear Pim-1 expression already in the primary diagnostic biopsy sample. By contrast, irradiation induced nuclear translocation of Pim-1 immunoreactivity in the rest of the tumors (8/17) that, before irradiation, demonstrated preferentially cytoplasmic localization of Pim-1 (P < .005). These data suggest that irradiation induces nuclear translocation of Pim-1 in tumors, which do not respond well to irradiation.

Ligand-Induced Activation of EGFR Regulates Pim-1 Expression and Subcellular Localization

Our results demonstrated that Pim-1 and EGFR expression associated with each other in HNSCC. In addition, we showed that both Pim-1 and EGFR associated with poor response to irradiation and that Pim-1 was translocated into the nuclei of HNSCC cells after irradiation in radioresistant tumors. To discriminate whether the expression patterns of EGFR and Pim-1 are simply coincidental or whether these molecules are involved in the same signaling pathway in HNSCC, we next studied the putative effects of EGFR activation on Pim-1 expression and subcellular localization using previously established HNSCC cell lines expressing Pim-1 and EGFR (Table 2). Although the cell lines were originally grown from human HNSCC, we first wanted to validate the use of these cell lines by testing their capacity to still form tumors *in vivo*. The selected cell lines (UT-SCC-12A, UT-SCC-22, UT-SCC-40, UT-SCC-43A, and UT-SCC-54C; Table 2) represented tumors, which originated from different anatomical locations, were of different grades

Table 1. Correlations to Radiation Response.

Parameter	*P*
Age	.65
Sex	.53
Site of primary tumor	.98
Tumor size	.19
Primary clinical stage	.28
Nodal status	.50
Histological grade	.44
Overall survival	**.05**
EGFR positivity	**.018**
Pim-1 positivity	**.003**

P values in bold face font are statistically significant.

Table 2. Characteristics of the Patients and Specimens Used to Establish the HNSCC.

Cell Line	Sex	Primary Tumor	TNM Location	Specimen	Type of Site	Grade* Lesion
UT-SCC-8	M	Supraglottic larynx	T2 N0 M0	Larynx	pri	G1
UT-SCC-12A	F	Skin of the nose	T2 N0 M0	Skin	pri	G1
UT-SCC-19A	M	Glottic larynx	T4 N0 M0	Larynx	pri	G2
UT-SCC-22	M	Glottic larynx	T1 N0 M0	Larynx	rec	G2
UT-SCC-24A	M	Tongue	T3 N0 M0	Tongue	pri	G2
UT-SCC-32	M	Tongue	T3 N0 M0	Oral tongue	pri (per)	G1
UT-SCC-38	M	Glottic larynx	T2 N0 M0	Larynx	pri	G2
UT-SCC-40	M	Tongue	T3 N0 M0	Tongue	pri	G1
UT-SCC-43A	F	Gingiva of mandible	T4 N1 M0	Gingiva of mandible	pri	G2
UT-SCC-50	M	Glottic larynx	T2 N0; rT2 N0	Larynx	rec	G3
UT-SCC-54C	F	Buccal mucosa		Neck	met	G1

met indicates metastasis; *pri*, primary tumor; *pri (per)*, primary tumor, persistent after radiotherapy; *rec*, recurrence.
*Grade of the original tumor.

and had different intracellular localization of Pim-1 when grown *in vitro* (Tables 2 and 3). A spontaneously transformed HaCaT human epithelial cell line with full differentiation potential was used for comparison. All cell lines were injected subcutaneously into SCID mice. Whereas HaCaT cells reaggregated and formed small rounded nests with multilayered sheaths of keratinocytes, all five HNSCC cell lines grew rapidly at the injection sites, and the animals had to be killed 25 to 41 days after injection owing to rapid tumor growth. The expression pattern of Pim-1 in HaCaT cells *in vivo* was rather diffuse, concentrating mostly in the cytoplasm (Figure 3*A*) and resembling Pim-1 expression in cultured HaCaT cells and in normal skin during keratinocyte differentiation (data not shown). In all HNSCC cell lines grown *in vivo*, nuclear Pim-1 expression was observed (two examples shown in Figure 3*A*). These findings indicate that the cell lines selected for our analyses reproduced the same ability to translocate Pim-1 into the nucleus as observed in the clinical HNSCC samples *in vivo*.

Although it is known that irradiation causes even more long-lasting internalization of EGFR than EGF [22], it may cause many other effects as well. Therefore, we used specific EGFR ligands to directly test whether EGFR activation regulates expression levels or distribution of Pim-1. The *in vitro* cultured HNSCC lines were stimulated with EGF and TGF-α. Treatments with either EGF or TGF-α resulted in the internalization of EGFR (Figure W1) and in an increase of Pim-1 expression levels in all HNSCC cell lines (Figure 3*B* and Table 3). Furthermore, EGF and TGF-α induced translocation of Pim-1 into the nucleus in 10 of 11 HNSCC lines studied (Figure 3*B* and Table 3). Nuclear translocation took place in more than 80% of the cells, both in confluent and subconfluent cell cultures, indicating that translocation

Table 3. Subcellular Localization of Pim-1 Expression in the HNSCC Cell Lines after Different EGFR Stimulation and Inhibition Treatments.

Cell Line	Treatment			
	–	EGF	EGF + Inh*	TPA
UT-SCC-8	Double pos†++	More nuclear +++	Double pos++	ND‡
UT-SCC-12A	More cytopl§++	Nuclear+++	More cytopl++	More cytopl++
UT-SCC-19A	Double pos++	More nuclear+++	Double pos++	ND
UT-SCC-22	Double pos++	Nuclear+++	Double pos++	More cytopl++
UT-SCC-24A	Double pos+	Nuclear+/++	Double pos+/++	ND
UT-SCC-32	Double pos++	Nuclear++/+++	Double pos++	Cytopl++
UT-SCC-38	More cytopl+	Nuclear++	Cytopl+	ND
UT-SCC-40	Cytopl+	Cytopl++	Cytopl+	Cytopl+
UT-SCC-43A	Double pos++	More nuclear+++	Double pos++	More nuclear++
UT-SCC-50	Double pos++	More nuclear+++	Double pos++	Cytopl++
UT-SCC-54C	Double pos++	More nuclear+++	Double pos++	More cytopl+/++

*Inh indicates gefitinib and cetuximab (both treatments were separately combined to the EGF treatment with comparable results).
†Double positive, comparable level of expression in cytoplasm and nucleus.
‡ND indicates not determined.
§Cytopl indicates cytoplasmic.

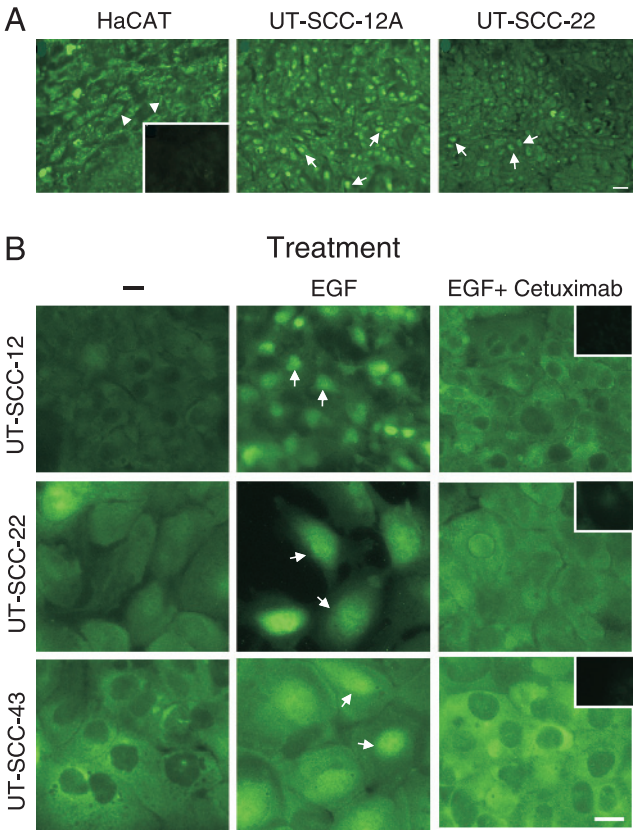

Figure 3. The HNSCC cells form tumors in SCID mice with mostly nuclear Pim-1 expression, and the nuclear translocation of Pim-1 is further promoted by EGFR activation. Cells were stained with anti–Pim-1 antibody and analyzed under immunofluorescence microscope. (A) HaCaT cells (used as controls) show mostly diffuse cytoplasmic staining (arrowheads), whereas UT-SCC-12A and UT-SCC-22 display preferentially nuclear Pim-1 expression (arrows). HaCaT cells stained with a negative control antibody are shown in the inset. Bar, 50 μm. (B) Expression of Pim-1 in cell lines UT-SCC-12A, UT-SCC-22, and UT-SCC-43A was analyzed after indicated treatments. Arrows indicate the nuclear translocation of Pim-1. Stainings with a negative control antibody are shown in the insets. Bar, 20 μm.

was not cell cycle–dependent. By contrast, nuclear translocation of Pim-1 was observed in only one HNSCC cell line treated with the phorbol ester TPA (Table 3), demonstrating specificity of the effect. The EGF-induced nuclear translocation of Pim-1 in HNSCC cell lines could be inhibited with an anti-EGFR antibody, cetuximab (Figure 3*B*) as well as with EGFR targeting tyrosine kinase inhibitor, gefitinib (data not shown). These data indicate that ligand-induced activation of EGFR by EGF or TGF-α is sufficient to induce nuclear translocation of Pim-1 in HNSCC cell lines.

Irradiation Induces EGFR-Dependent Nuclear Translocation of Pim-1 and Pim-1 Is Involved in Subsequent Cell Survival

To test whether irradiation causes the nuclear translocation of Pim-1, we subjected SCC-12, SCC-22, and SCC-43 cell lines to 2 Gy irradiation that corresponds to the daily dose given to patients. Indeed, this treatment triggered nuclear localization of Pim-1 that could be inhibited by pretreatment with EGFR inhibitors, cetuximab or gefitinib (examples shown in Figure 4*A* and the situation without irradiation is seen in Figure 3*B*). These data confirm that irradiation can cause nuclear translocation of Pim-1 in an EGFR-dependent manner.

Next, we evaluated whether presence of Pim-1 affected the survival of cancer cells after irradiation. Before irradiation, the cell lines were treated with Pim-1–specific siRNA (Pim-siRNA) or NT control siRNA. The Pim-siRNA treatment efficiently diminished Pim-1 expression (Figure 4*B*), and the cells with the remaining low levels of Pim-1 expression survived significantly poorer after irradiation than cells treated with NT siRNA and retaining good Pim-1 expression levels. Trypan blue and flow cytometric analyses with ModFit program gave comparable results. The results with trypan blue are shown in Figure 4*B*. These results support the concept that Pim-1 protects the cells from irradiation damage.

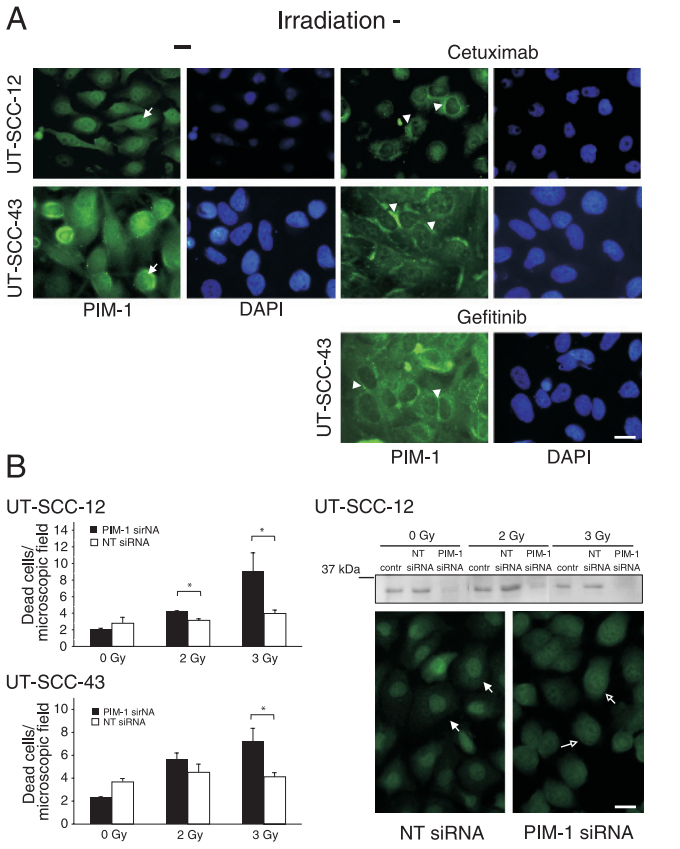

Figure 4. Irradiation induces EGFR-dependent nuclear translocation of Pim-1, and Pim-1 is involved in subsequent cell survival. (A) Pim-1 expression in UT-SCC-12 and UT-SCC-43 cells 2 days after irradiation without and with pretreatment of cetuximab and gefitinib (UT-SCC-43). Nuclear staining with 4′, 6-diamidino-2-phenylindole. Some cells with nuclear Pim-1 expression are pointed out by arrows, and those with cytoplasmic Pim-1 expression are indicated with arrowheads. (B) UT-SCC-12 and UT-SCC-43 cell lines were treated with Pim-1–specific siRNA or NT siRNA before irradiation. Combined results of the cell survival after the treatments are presented: $n = 3$. The effects of siRNA on Pim-1 expression are shown for SCC-12 in Western blot and immunofluorescence stainings after 2 Gy irradiation with anti–Pim-1 antibody followed by fluorescein isothiocyanate–conjugated second-stage antibody. Closed arrows point to nuclear Pim-1 positivity in NT siRNA-treated cells, and open arrows point to dimmer Pim-1 staining both in the cytoplasm and in the nucleus of partially rounded and detached cells treated with Pim-1 siRNA. Bar, 20 μm.

Discussion

Here, we describe that the Pim-1 kinase is overexpressed in malignant keratinocytes in HNSCC. In addition, we demonstrate that moderate/high Pim-1 expression levels tend to associate with poor prognosis in HNSCC compared with low/negative Pim-1 expression levels. Importantly, Pim-1 overexpression significantly associates with a poor response to radiotherapy. Because the classic prognostic markers such as tumor size, nodal status, or histological grade do not predict response to radiotherapy, and there are currently no generally accepted clinical markers predicting radiosensitivity, these results may have clinical implications. Expression of Pim-1 also significantly associates with expression of EGFR, which has previously been demonstrated to contribute to radiation resistance [19].

Myeloid and lymphoid malignancies as well as prostate cancer often display increased levels of Pim-1 [14,15]. It has been suggested that Pim-1 overexpression drives genomic instability in early tumors, whereas advanced tumors may downregulate Pim-1 and thereby stabilize acquired abnormalities [23]. Moreover, Pim-1 has been identified as a survival factor for myeloid cells deprived of cytokines [1,2] or subjected to genotoxic stress [24]. Thus, HNSCC cells expressing excessive amounts of Pim-1 may be protected against cell death and are thereby also resistant to irradiation. These conclusions are consistent with the enhanced carcinogenesis observed in *pim-1* transgenic mice subjected to irradiation [25].

Owing to heterogeneity in the intracellular localization of Pim-1 protein within individual HNSCC tumors, it was practically impossible to take subcellular localization into consideration when analyzing the primary diagnostic samples. However, we were able to show that irradiation promoted Pim-1 translocation into the nuclei of HNSCC cells in radioresistant surgical samples. Furthermore, recent data by Chiang et al. [26] together with our current data indicate that Pim-1 is mostly cytoplasmic in cells of normal squamocellular epithelium such as tonsil and skin, whereas its expression is upregulated and predominantly nuclear in many malignant cells. We assume that nuclear localization of Pim-1 is required for its full activity, especially for its ability to regulate several transcription factors and cell cycle regulators. In accordance, nuclear localization of Pim-1 has been shown to be necessary for its biological effects in Burkitt lymphoma [21].

Enhanced expression of Pim-1 was also detected in HNSCC-derived cell lines established from the original patient samples when compared

with normal human keratinocytes. Interestingly, as shown by immunofluorescence analyses, Pim-1 expression levels were upregulated by ligand-induced activation of EGFR. Furthermore, activation of EGFR efficiently shifted the localization of Pim-1 from the cytoplasm to the nucleus. Most likely *in vivo* conditions provide sufficient amounts of EGFR ligands because in EGFR-expressing HNSCC cell lines grown as tumors in SCID mice, Pim-1 protein is predominantly nuclear.

Epidermal growth factor receptor is one of the upstream activators of Signal Transducer and Activator of Transcription 3 and Signal Transducer and Activator of Transcription 5, and *pim-1* is a target gene for both of them [8,27]. Therefore, these STATs are the prime candidates to mediate the signal from EGFR to Pim-1. On the basis of structural studies, the Pim-1 kinase is considered to reside in a constitutively active conformation [28]. When its expression and thereby also activity is upregulated, Pim-1 promotes progression in the cell cycle and inhibits apoptosis by phosphorylating many key enzymes involved in these processes [8]. Enhanced EGFR signaling due to elevated EGFR expression levels is also linked to cell proliferation and survival, leading to tumor progression and decreased survival of patients with HNSCC [29]. Interestingly, irradiation and stress are known to lead to activation and intracellular translocation of EGFR, and their effects are even more persistent than that caused by EGF [22]. Overexpression and activation of STAT3 and STAT5 are also common in HNSCC [30], indicating that perturbations in any of these signaling steps can have similar consequences. In this context, it is interesting to note that inhibition of EGFR increases intrinsic radiosensitivity of HNSCC cell lines *in vitro* [31] and that monoclonal antibody therapy against EGFR improves locoregional control and survival of patients with head and neck cancer, when combined with radiotherapy [32,33]. On the basis of the existing literature and our current findings, we suggest that irradiation causes activation of EGFR and its simultaneous intracellular translocation leading to nuclear translocation of Pim-1 and up-regulation through STAT3/5 as depicted in Figure 5. Thus, the efficacy of therapeutics targeting EGFR in HNSCC may be due to the inhibition of EGFR-mediated signals activating and translocating Pim-1.

On the basis of our data, HNSCC patients with tumors expressing moderate or high levels of Pim-1 may not be primary candidates for radiation therapy. Nevertheless, in the future, radioresistant HNSCCs could be targeted with inhibitors against Pim-1. Lead compounds for developing such inhibitors have already been identified [34]. Moreover, because Pim-1 can receive regulatory signals also through other receptors besides EGFR and even from cytotoxic drugs such as docetaxel [35], targeting of EGFR and Pim-1 simultaneously may provide synergistic therapeutic effects when treating primarily radioresistant tumors.

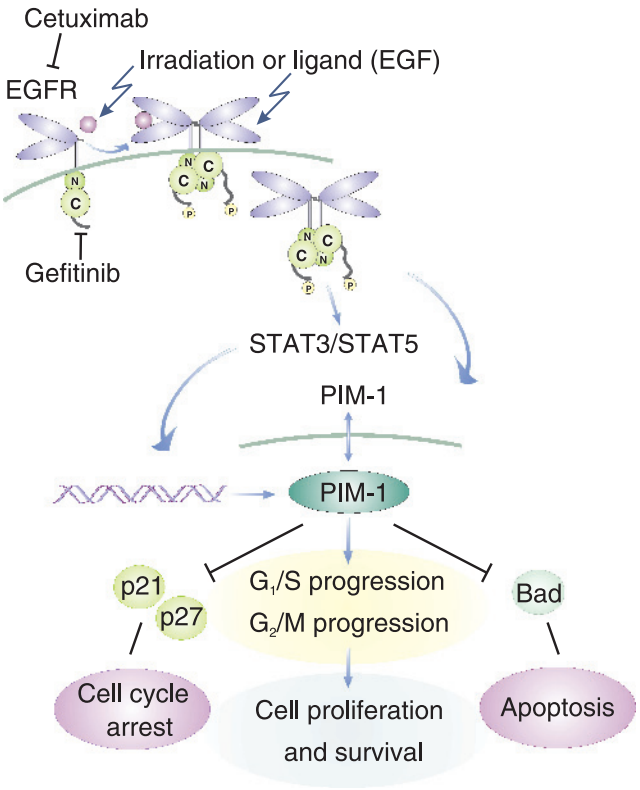

Figure 5. A schematic model depicting the connection between EGFR and Pim-1 in HNSCC. Irradiation (and ligand binding) causes dimerization and intracellular translocation of EGFR. The signal is further transmitted through STAT3 and STAT5 to Pim-1 that is activated and translocated into the nucleus. Moreover, STAT3 and STAT5 can directly induce Pim-1 transcription. On the basis of the existing literature, increased amount of Pim-1 has a multitude of fundamental effects determining tumor growth as indicated in the figure. Other possible EGFR-signaling pathways have been omitted for simplicity.

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Table W1. Pretreatment Characteristics of the Patients with HNSCC.

Parameter	*N*	%
Sex		
M	41	57.8
F	30	42.2
Tumor size		
T1	14	19.7
T2	31	43.7
T3	16	22.5
T4	10	14.1
Primary clinical stage		
Stage I	11	15.5
Stage II	27	38.0
Stage III	17	23.9
Stage IV	16	22.6
Nodal status		
N0	50	70.4
N1	13	18.3
N2	8	11.3
Histological grade		
1	35	49.3
2	27	38.0
3	9	12.7
Location		
Larynx	2	2.8
Tongue	20	28.2
Other oral cavity	23	32.4
Tonsil	4	5.6
Hypopharynx	12	16.9
Other	10	14.1

Table W2. Treatment and Follow-up Characteristics.

Follow-up time of patients (months)	
Median	24
Range	2.0-86
Radiation therapy, *n* (%)	
Preoperative (60-70 Gy)	45 (63.4)
Definitive curative	18 (25.4)
Postoperative	1 (1.4)
Palliative	1 (1.4)
No radiation therapy	6 (8.5)
Surgery, *n* (%)	
Curative	50 (70.4)
Palliative	2 (2.8)
No operation	19 (26.8)
Chemotherapy	None
Local recurrence, *n* (%)	26 (36.6)
Persisting disease, *n* (%)	13 (18.3)

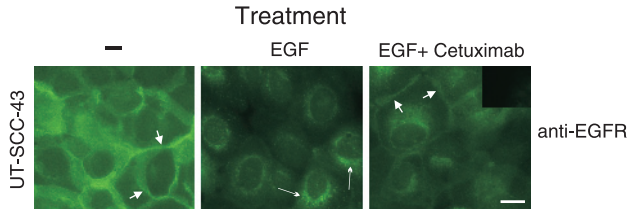

Figure W1. Epidermal growth factor receptor is internalized after treatment with EGF and is inhibited by cetuximab. UT-SCC-43 cell line was stained after different treatments with the anti-EGFR antibody, as indicated in the figure. Note the cell surface localization on nontreated cells (left panel, pointed out by short arrows), cytoplasmic localization after EGF treatment (middle panel, longer arrows), and partial inhibition of internalization in the presence of cetuximab. Positivity on the cell surface is pointed out by short arrows (right panel). Bar, 20 μm.