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Activation of the insulin-like growth factor-1 receptor alters p27 regulation by the epidermal growth factor receptor in oral squamous carcinoma cells

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Abstract

Background—Although oral squamous cell carcinomas (OSCCs) commonly overexpress the epidermal growth factor receptor (EGFR), EGFR tyrosine kinase inhibitors (TKIs) exhibit poor efficacy clinically. Activation of the IGF1R induces resistance of OSCC cells to EGFR-TKIs *in vitro*. This study seeks to evaluate the changes in cell cycle status in OSCC cells in response to gefitinib and IGF1R activation.

Methods—SCC-25 OSCC cells were used for *in vitro* analyses.

Results—Gefitinib caused a 50% reduction in S-phase population and IGF1R activation caused a 2.8-fold increase; combined treatment yielded a baseline S-phase population. Gefitinib treatment increased the cyclin-dependent kinase inhibitor p27 and this was not abrogated by IGF1R activation. pT157-p27 was noted by immunoblot to be decreased on gefitinib treatment, but this was reversed with IGF1R activation. T157 phosphorylation contributes to cytoplasmic localization of p27 where it can promote cell proliferation and cell motility. Using both subcellular fractionation and immunofluorescence microscopy techniques, IGF1R stimulation was noted to increase the relative cytoplasmic localization of p27; this persisted when combined with gefitinib.

Conclusions—IGF1R activation partially reverses the cell cycle arrest caused by gefitinib in OSCC cells. While IGF1R stimulation does not eliminate the gefitinib-induced increase in total p27, its phosphorylation state and subcellular localization are altered. This may contribute to the ability of the IGF1R to rescue OSCC cells from EGFR-TKI treatment and may have important implications for the use of p27 as a biomarker of cell cycle arrest and response to therapy.

Keywords

oral cavity cancer; therapeutic resistance; cell cycle regulation; growth factor; cyclin-dependent kinase inhibitor

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INTRODUCTION

The epidermal growth factor receptor (EGFR) is an established therapeutic target in head and neck squamous cell carcinoma (HNSCC). Several targeted anti-EGFR agents have been developed, but their efficacy in HNSCC is limited due to frequent intrinsic or acquired resistance. To date, molecular markers that predict sensitivity of HNSCC to anti-EGFR drug have not been identified.(1) However, EGFR inhibition in HNSCC is known to cause cell cycle arrest which is dependent on increased p27 expression.(2)

p27 is a cell cycle regulator that was initially identified as a CDK inhibitor of the cyclin E/CDK2 complex.(3) It mediates cell cycle arrest by impairing the ability of cyclin E to promote G1-S transition. This function is regulated primarily by the amount of nuclear p27; p27 degradation via ubiquitination causes increased cyclin E activity and cell cycle progression. Thus, p27 expression has been interpreted as a surrogate for overall cell cycle status. Studies of HNSCC have correlated greater disease burden and/or poorer survival outcomes with decreased p27 expression at multiple subsites including oral cavity (OSCC), (4–6) oropharynx,(6) larynx,(7) and hypopharynx.(8).

More recently, detailed study of p27 has demonstrated additional functional roles and highly complex regulation. p27 can inhibit or promote cell cycle progression based on protein level and subcellular localization (for review, see Wander *et al.*).(9) Serine, threonine, and tyrosine phosphorylation of p27 regulate its protein-protein interactions, nuclear import/export, and degradation, thus impacting its function. Phosphorylation at threonine-157 (pT157) results in accumulation in the cytoplasm where p27 interacts with cyclin D/CDK4 and RhoA to increase cell cycle progression and cell migration, respectively; in the setting of malignancy, these could portend therapeutic resistance and metastasis.(10;11) Thus, the interpretation of changes in total p27 expression in response to treatment with a targeted therapeutic agent is unlikely to correlate directly with biologic outcome.

We have recently demonstrated that, in OSCC cell lines, activation of the insulin-like growth factor-1 receptor (IGF1R) can overcome the growth inhibition caused by EGFR tyrosine kinase inhibitors (TKIs).(12) Although there is no established marker of this phenomenon, IGF1R-induced resistance to EGFR-TKIs was associated with increased Akt activity and reduced apoptosis. Noting that (1) the growth inhibitory (nuclear) function of p27 is required for EGFR-TKI efficacy, (2) IGF1R activation causes resistance to EGFR-TKIs, (3) the IGF1R is a potent activator of Akt, and (4) Akt phosphorylates p27 at T157 with resultant cytoplasmic sequestration of p27 and cell cycle progression, we evaluated regulation of p27 by EGFR-TKIs in an OSCC cell line in the presence or absence of simultaneous IGF1R activation.

MATERIALS AND METHODS

Reagents

des[1–3]IGF-1 was obtained from GroPep (Adelaide, Australia), EGF from Sigma (St. Louis, MO), PD158780 from EMD Biosciences (San Diego, CA), and gefitinib from LC Laboratories (Woburn, MA). Anti-IGF1R α was obtained from Santa Cruz Biotechnology, anti-pERK from Sigma, and anti-Akt, anti-pAkt (S473), anti-ERK, anti-pIGF1R, and anti-EGFR from Cell Signaling Technology (Beverly, MA).

Tissue Culture

SCC-25 cells were obtained from ATCC (Manassas, VA). They were grown in D-MEM/F12 supplemented with 400 ng/mL hydrocortisone and 5% FBS at 37C and 5% CO₂. *In vitro*, cells were histopathologically consistent with OSCC and were positive for cytokeratin.

Monolayers were grown to 70% confluence and starved in very low (0.5%) serum for 24 h before assays were performed. Cells were passaged for fewer than 6 months after resuscitation.

CellTiter 96® Aqueous Cell Proliferation Assay

Cells were plated at 5,000 cells/well in 96-well plates, grown for 24 h, serum-starved for 24 h, and treated with inhibitor/growth factor for 48 h. MTS and PMS were added according to the manufacturer's protocol (Promega, Madison, WI). Plates were incubated for 4 h at 37C and the absorbance at 490 nm was recorded.

AlamarBlue® Assay

Cells were prepared and treated as above. AlamarBlue (Invitrogen, Carlsbad, CA) was added according to the manufacturer's protocol. Plates were incubated for 3–4 h at 37C and the fluorescence at 540 nm was recorded.

Flow Cytometry

For standard propidium iodide cell cycle analysis, cell monolayers were assessed after 48 h of treatment. Cells were trypsinized, resuspended in the corresponding medium, pelleted, washed, and resuspended in propidium iodide according to the manufacturer's protocol (Millipore). Flow cytometry was performed in the UVA Flow Cytometry Core Facility within 24 h. For the "triple stain protocol" (Invitrogen) which quantifies cell cycle, DNA synthesis, and apoptosis, cell monolayers were assessed after 24 h of treatment according to the manufacturer's protocol. BrdU (Invitrogen) was added (10 µg/mL final) and incubation continued for 6 h. Cells were trypsinized, resuspended in the corresponding medium, counted, pelleted, resuspended in ice cold phosphate-buffered saline (PBS)/ethanol, and stored at –20C. At the time of flow cytometry, 2 million cells were resuspended sequentially in incubation buffer, HCl, phosphate/citrate buffer, and incubation buffer. Cells were sequentially incubated at RT in anti-cleaved caspase-3 (Cell Signaling; 1:1,600) and anti-BrdU antibodies (BD Pharmingen; 1:250) for 1 h, R-phycoerythrin goat anti-rabbit IgG (1:300) for 30 min, and TO-PRO®-3 stain (Invitrogen) for 45 min. Flow cytometry was performed immediately.

Immunoblot

Starved monolayers were washed with PBS and incubated with inhibitor or vehicle for 2 h followed by growth factor for 15 min. Treated monolayers were washed with ice cold PBS containing 2 mM sodium orthovanadate, collected, and lysed. In some experiments, cytoplasmic and nuclear fractions were extracted using the Pierce Subcellular Fractionation Kit (Thermo Scientific, Rockford, IL). Equivalent protein from each sample was subjected to SDS-polyacrylamide gel electrophoresis, semi-dry transfer, and immunoblotting as previously described.(12)

Immunofluorescence Microscopy

Cells were grown and treated on coverslips. After 24 h of treatment, cells were fixed with 4% formaldehyde in PBS for 15 min at RT. Coverslips were washed in PBS and incubated in blocking buffer (5% normal goat serum, 0.1% saponin in PBS) for 60 min at RT followed by anti-p27 antibody (Cell Signaling) in antibody dilution buffer (1% BSA, 0.1% saponin in PBS) overnight at 4C. After washing, coverslips were incubated in fluorochrome-conjugated anti-Rabbit IgG antibody in antibody dilution buffer for 2 h at RT in darkness and nuclei were counterstained with DAPI according to the manufacturer's instructions. Microscopy was performed immediately. Image exposure conditions were standardized using the DAPI stain.

RESULTS

IGF1R-Induced Resistance to EGFR-TKIs Correlates with Partial Reversal of Cell Cycle Arrest

Activation of the IGF1R was tested for its ability to alter OSCC sensitivity to EGFR-TKIs (Fig. 1). IGFBP-2 and -3 are secreted at high levels by some OSCC cells,(13) and can interfere with activation of the IGF1R by exogenously added free IGF-1. Because of its dramatically reduced affinity for the IGFBPs, des[1–3]IGF-1 was used to activate the IGF1R.

SCC-25 cells treated with gefitinib for 48 h (Fig. 1A) show a dose-dependent reduction in cell number. Activation of the IGF1R with des[1–3]IGF-1 eliminated the growth-inhibitory effect of gefitinib below 10 μ M. Similarly, PD158780 reduced cell number by 66% at 48 h (Fig. 1B). Addition of exogenous EGF increased cell number by 40% and this effect was blocked by PD158780. IGF1R activation minimally increased cell number and this effect was not blocked by PD158780. FBS stimulated resulted in an 86% increase in cell number; this was partially blocked by PD158780 (Fig. 1B). These results demonstrate that, in SCC-25 cells, activation of the IGF1R confers resistance to the growth inhibitory effects of EGFR inhibition.

Figure 2A shows a representative flow cytometric analysis of SCC-25 cells treated with gefitinib and/or des[1–3]IGF-; Figure 2B shows a graphic summary of 3 similar independent experiments. Gefitinib caused a statistically significant decrease ($p < 0.05$) in the basal S-phase population from 21% to 5% and IGF1R activation produced a statistically significant increase ($p < 0.05$) in the S-phase population to 33%. The combination of EGFR inhibition and IGF1R activation resulted in an S-phase population of 12%, which was not significantly different from baseline, but was significantly different ($p < 0.05$) from cells treated with gefitinib alone. Similar results were obtained with PD158780 (Fig. 2C).

IGF1R-Induced Resistance to EGFR-TKIs Correlates with Persistent Akt Activation and Cyclin D Expression, But Not Reduced p27 Expression

The effects of gefitinib (Fig. 3A) and PD158780 (Fig. 3B) on signaling molecules downstream of the EGFR and IGF1R in SCC-25 cells were assessed by immunoblot analysis. Treatment with either EGFR-TKI reduced or eliminated basal Akt and ERK phosphorylation. Stimulation with EGF caused ERK activation that was also inhibited by both drugs. Activation of the IGF1R resulted in prominent Akt activation and less substantial ERK activation in uninhibited cells. After pretreatment with gefitinib or PD158780, IGF-induced ERK activation was eliminated but IGF-induced Akt activation was unaffected. This indicates EGFR dependence of IGF1R-induced ERK activation as has been previously reported (13) and demonstrates that Akt activation persists when the IGF1R is activated in the presence of EGFR antagonists.

Levels of pRb, cyclin D, and p27 were assessed by immunoblot after treatment with gefitinib (Fig. 4A) or PD158780 (Fig. 4B) as markers of cell cycle status. We have previously correlated higher cyclin D expression with poorer survival in HNSCC.(14) Treatment with either EGFR-TKI resulted in decreased Rb phosphorylation as indicated by reduced band density using a phospho-specific antibody (Fig. 4A) and by reduced band shift (Fig. 4B). These drugs also caused reduced cyclin D expression and increased p27 expression. Treatment with des[1–3]IGF-1 resulted in Rb phosphorylation as indicated by increased band density using a phospho-specific antibody (Fig. 4A) and by increased band shift (Fig. 4B). IGF1R activation also increased cyclin D expression and did not impact baseline p27 expression. When EGFR inhibition was combined with IGF1R activation, Rb phosphorylation was reduced compared to treatment with des[1–3]IGF-1 alone, cyclin D

expression remained elevated, and p27 expression also remained elevated. These data indicate that, with EGFR inhibition in the context of IGF1R activation, p27 is persistently elevated despite high cyclin D expression, an S-phase population similar to untreated cells (Fig. 2) and continued cell proliferation (Fig. 1).

IGF1R Activation Reverses Changes in p27 Phosphorylation and Subcellular Localization Caused by EGFR Inhibition

Figure 5A shows a representative immunoblot of p27 and pT157-p27 in SCC-25 cells treated with gefitinib and/or des[1–3]IGF-1. Figures 5B and 5C, respectively, show graphic analyses of band densities for total p27 and pT157-p27. Treatment of SCC-25 cells with gefitinib resulted in a statistically significant increase in total p27 expression and a concurrent significant reduction in T157 phosphorylation. IGF1R activation had no effect on basal total p27 levels or degree of T157 phosphorylation. When gefitinib and des[1–3]IGF-1 treatments were combined, total p27 was significantly increased compared to untreated cells (similar to gefitinib alone), but pT157-p27 remained at basal levels, which was significantly different from gefitinib treatment alone.

Figure 6 shows immunofluorescence microscopy of SCC-25 cells using an anti-p27 antibody. Compared to untreated cells, gefitinib treatment increased and des[1–3]IGF-1 treatment decreased nuclear p27 levels while the combination most closely resembled untreated cells. Immunoblot analysis of subcellular fractions (Fig. 7A) correlated well. For total p27 (Fig. 7B), subcellular fractionation demonstrated a reduced cytoplasmic to nuclear ratio (CNR) with gefitinib treatment alone, an increased CNR with IGF1R activation alone, and a baseline CNR with both treatments combined. For pT157-p27 (Fig. 7C), gefitinib treatment did not significantly reduce the CNR but it was significantly enhanced by IGF1R activation alone; the effect of IGF1R activation persisted in the presence of gefitinib.

DISCUSSION

The present study further supports the emerging concept that evaluation of the subcellular localization and phosphorylation status of p27 are crucial to its use as a biomarker in OSCC. While initial observations of p27 suggested that total p27 expression detected by immunoblot or immunohistochemistry (IHC) could report cell cycle status and thus the response to intervention, subsequent investigations have cast doubt on this theory. The present data clearly show that, in SCC-25 cells, EGFR-TKIs cause cell cycle arrest in G1 and that this is accompanied by increased total p27, with more intense nuclear localization than uninhibited cells. This correlates with a significant decrease in p27 phosphorylation at T157. In contrast, activation of the IGF1R with exogenous ligand in the absence of EGFR has little impact on the basal p27 expression or basal T157 phosphorylation. However, with simultaneous EGFR inhibition and IGF1R activation, T157 phosphorylation remains at baseline levels (significantly increased compared to cells treated with gefitinib alone) while total p27 increases. This results in cytoplasmic retention of p27 with reduced nuclear localization and no cell cycle arrest relative to untreated cells.

As suggested by Wander et al.,⁽⁹⁾ cytoplasmic retention of p27 that is newly synthesized in G1 could be facilitated by effectors downstream of phosphatidylinositol-3'-kinase (PI3K) that phosphorylate p27 at T157; this would be expected to promote cyclin D/CDK4/p27 assembly, leading to some degree of cell cycle progression. In the case of SCC-25 cells, we speculate that, in the absence of IGF1R activation, inhibition of the EGFR results in increased cytoplasmic p27 synthesis in the context of low PI3K/AKT activity such that T157 phosphorylation is low and the newly synthesized p27 predominantly undergoes nuclear import and interacts with cyclin E/CDK2 to facilitate G1 arrest. Based on this proposed mechanism, when the IGF1R is activated and PI3K/AKT activity is high, we would expect

p27 being synthesized due to EGFR inhibition to undergo more T157 phosphorylation resulting in more cytoplasmic retention and less cell cycle arrest; we have demonstrated both phenomena in the present study. PI3K/AKT activation, as is present in SCC-25 cells treated with des[1–3]IGF-1, is highly correlated with cytoplasmic p27 localization, reduced CDK2 inhibitory activity, and cell cycle progression in other cell types.(11;15–17) Although not within the scope of this study, we anticipate that, under conditions of IGF1R activation, SCC-25 cells treated with EGFR-TKIs retain some characteristics of invasive and metastatic behavior that are consistent with increased cytoplasmic p27,(9) further supporting the notion that IGF1R may be an important target for therapy in OSCC. This warrants further investigation in future studies.

Decreased or “mislocalized” p27 as detected by IHC has been associated with poor outcomes in multiple cancer types being studied *in vitro*.(18) However, to date, the primary focus of clinically-related p27 assessment in HNSCC has been on retrospective evaluation of p27 as a pre-treatment prognostic marker.(4–8) In this context, many studies have evaluated nuclear p27 since it appears to correlate most closely with outcomes, but staining protocols are inconsistent. Indeed, some studies of OSCC have found no correlation;(19) it is possible that these findings are a result of inclusion of cytoplasmic staining, which highlights the need for more precise assessment of p27 status. Increased cytoplasmic p27 is less studied by appears to correlate with poorer outcomes in some cancers, and few studies prior to the present work have evaluated both nuclear and cytoplasmic p27 simultaneously. (18) In the context of predicting outcomes of targeted therapy, p27 is likely to be useful, but will need to be assessed prospectively and dynamically (i.e., changes with treatment) with attention to both subcellular localization and phosphorylation status.

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Figure 1. Inhibition of SCC-25 proliferation by EGFR-TKIs and rescue by IGF1R activation
 (A) SCC-25 cells were incubated for 48 h with gefitinib or vehicle $\pm$ 10 nM des[1–3]IGF-1 and assessed using the AlamarBlue assay. Graph shows average percentage of uninhibited/unstimulated cells $\pm$ SEM for 3 independent quadruplicate experiments. (B) SCC-25 cells were incubated for 48 h with 500 nM PD158780 and the additional treatments shown and assessed using the MTS assay. Graph shows average fold change compared to uninhibited/unstimulated cells $\pm$ SEM for 4 independent quadruplicate experiments.

Figure 2. Effect of EGFR-TKIs and IGF1R activation on cell cycle

A–B, SCC-25 cells were incubated for 24 h with gefitinib or vehicle $\pm$ 10 nM des[1–3]IGF-1 and assessed using flow cytometry. (A) Representative FACS scatter plots showing BrdU incorporation vs. TO-PRO-3 nucleic acid staining. (B) Bar graph representing average % S-phase in 3 independent experiments $\pm$ SEM, * $p < 0.05$ compared to uninhibited/unstimulated cells, ** $p < 0.05$ compared to cells treated with gefitinib only (not significantly different vs. uninhibited/unstimulated cells). (C) SCC-25 cells were incubated for 24 h with the growth factors indicated $\pm$ 500 nM PD158780. Cell cycle status was determined by flow cytometry using propidium iodide. Results are shown as fold change in % S-phase compared to uninhibited/stimulated cells. Data represent the mean $\pm$ SEM for 4 independent experiments.

Figure 3. Effect of EGFR-TKIs and IGF1R activation on ERK and Akt signaling
 SCC-25 cells were incubated for 2 h with gefitinib (A) or PD158780 (B) followed by stimulation with 5 nM EGF or 10 nM des[1-3]IGF-1 for 15 min. Whole cell lysates were collected and assessed by immunoblot with the indicated antibodies. Blots are representative of 3 independent experiments.

Figure 4. Effect of EGFR-TKIs and IGF1R activation on cell cycle regulators
 SCC-25 cells were incubated for 2 h with 1 μ M gefitinib (A) or 500 nM PD158780 (B) followed by stimulation with 5 nM EGF or 10 nM des[1-3]IGF-1 for 24 h. Whole cell lysates were collected and assessed by immunoblot with the indicated antibodies. Blots are representative of 3 distinct experiments.

Figure 5. Effect of gefitinib and IGF1R activation on total p27 and pT157-p27

SCC-25 cells were incubated for 2 h with gefitinib followed by stimulation with 5 nM EGF or 10 nM des[1-3]IGF-1 for 24 h. Whole cell lysates were collected and assessed by immunoblot with the indicated antibodies. (A) Representative immunoblot. (B) and (C) Densitometric analysis of total p27 and pT157-p27, respectively. Bars represent average fold change in band density compared to unstimulated/uninhibited cells $\pm$ SEM for 3 distinct experiments, * $p < 0.05$ compared to unstimulated/uninhibited cells, ** $p < 0.05$ compared to cells treated with gefitinib only (not significantly different vs. uninhibited/unstimulated cells).

Figure 6. Effect of gefitinib and IGF1R activation on p27 localization

SCC-25 cells were treated as indicated for 24 h and stained with DAPI and anti-p27 for immunofluorescence microscopy. Images shown are representative fields from two independent experiments that produced similar results. Gef = gefitinib; IGF = des[1–3]IGF-1.

Figure 7. Effect of gefitinib and IGF1R activation on p27 and pT157-p27 subcellular localization SCC-25 cells were incubated for 2 h with gefitinib followed by stimulation with 10 nM des[1-3]IGF-1 or vehicle for 24 h. Whole cell lysates were collected, fractionated as described in Methods, and assessed by immunoblot with the indicated antibodies. (A) Representative immunoblot. (B) Densitometric analysis of total p27 and (C) pT157-p27. Bars represent average fold change in the ratio of normalized band densities of cytoplasmic to nuclear fractions as compared to unstimulated/uninhibited cells $\pm$ SEM for 2 independent experiments, * $p < 0.05$ and ** $p < 0.01$ compared to unstimulated/uninhibited cells.