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Phosphorylation of PI3K regulatory subunit p85 contributes to resistance against PI3K inhibitors in radioresistant head and neck cancer

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ABSTRACT

Objectives: PI3K/Akt/mTOR pathway is commonly activated in most cancers and is correlated with resistance to anticancer therapies such as radiotherapy. Therefore, PI3K is an attractive target for treating PI3K-associated cancers. *Material and Methods*: We investigated the basal expression and the expression after treatment of PI3K inhibitor or Src inhibitor of PI3K/Akt pathway-related proteins in AMC-HN3, AMC-HN3R, HN30 and HN31 cells by performing immunoblotting analysis. The sensitivity to PI3K inhibitors or Src inhibitor was analyzed by MTT assay and clonogenic assay. To determine the antitumoral activity of combination treatment with PI3K inhibitor and Src inhibitor, we used using xenograft mouse model. *Results*: We found that PI3K regulatory subunit p85 was predominantly phosphorylated in radioresistant head and neck cancer cell line (HN31), which showed resistance to PI3K inhibitors. Next, we investigated mechanism through which PI3K p85 phosphorylation modulated response to PI3K inhibitors. Of note, constitutive activation of Src was found in HN31 cells and upon PI3K inhibitor treatment, restoration of p-Src was occurred. Src inhibitor improved the efficacy of PI3K inhibitor treatment and suppressed the reactivation of both Src and PI3K p85 in HN31 cells. Furthermore, downregulation of PI3K p85 expression by using a specific siRNA suppressed Src phosphorylation. *Conclusions*: Together, our results imply the novel role of the PI3K regulatory subunit p85 in the development of resistance to PI3K inhibitors and suggest the presence of a regulatory loop between PI3K p85 and Src in radioresistant head and neck cancers with constitutively active PI3K/Akt pathway.

Introduction

Phosphatidylinositol 3–kinase (PI3K)/AkT/mTOR axis regulates essential cellular functions, including cell metabolism, growth, migration, survival, and angiogenesis [1,2]. PI3K pathway activation is commonly observed in different cancers and is correlated with tumor development, progression, poor prognosis, and resistance to cancer therapies, including radiotherapy and chemotherapy [3–5]. Class IA PI3K is a heterodimeric protein containing a catalytic subunit (p110) and a regulatory subunit (p85). The p85 regulatory subunit binds to the p110 catalytic subunit through the iSH2 region and interacts with activated tyrosine kinases through the SH3 and SH2 domains, phosphotyrosine residues, and proline-rich motifs [6]. The PI3K p85 regulatory subunit is a lipid kinase that converts phosphatidylinositol (4,5)-bisphosphate to phosphatidylinositol (3,4,5)-trisphosphate (PIP3) in the plasma membrane, and PIP3 in turn activates downstream proteins such as AKT to promote cell growth and survival [7]. Binding of the p85 subunit to the p110 subunit stabilizes the holoenzyme PI3K and inhibits the catalytic activity of the p110 subunit until the binding of the p85 SH2 domain to phosphotyrosine peptides activates PI3K [8,9].

As a central node of the PI3K/Akt/mTOR pathway, PI3K is an attractive target for treating PI3K-associated cancers. Moreover, combination treatment regimens involving PI3K inhibitors may restore the sensitivity of cancers to other treatments [1,2,10]. Early clinical studies

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showed that both buparlisib and alpelisib had favorable tolerability profiles, "with the most common adverse event being "on-target" PI3K inhibition; however, the clinical efficacy of these drugs in single-agent settings has been modest thus far [11]. The complexity of the PI3K/AKT/mTOR pathway, which involves negative feedback loops, extensive crosstalk with other signaling pathways, and compensatory pathways, provides ample opportunities for the development of resistance to the PI3K inhibitors [10–14]. Investigation of these loops and pathways will help in completely realizing the potential of PI3K inhibitors in cancer treatment. Moreover, further studies are required to determine potential mechanism(s) underlying the development of resistance to PI3K inhibitors and to develop strategies to overcome the same.

Src belongs to membrane-associated non-receptor protein tyrosine kinase superfamily and is associated with the poor prognosis of patients with cancer. Importantly, Src regulates PI3K by directly phosphorylating its p85 regulatory subunit and by inhibiting the PI3K negative regulator PTEN [15]. Moreover, Src is recruited to and is activated in membrane compartments and phosphorylates the PI3K p85 regulatory subunit, thus increasing PI3K activity [9,16,17].

In the present study, we found that the PI3K regulatory subunit p85 was predominantly phosphorylated (at Tyr458) in radioresistant and PI3K inhibitor-resistant head and neck cancer (HNC) cells (HN31 cells). Notably, HN31 cells showed constitutive activation of Src; moreover, the phosphorylation of Src was maintained after PI3K inhibitor treatment. Treatment with Src inhibitor dasatinib improved the efficacy of PI3K inhibitor treatment and suppressed the reactivation of both Src and PI3K p85 in HN31 cells.

Materials and Methods

Cell culture and establishment of radioresistant HNC cell lines

This study included various HNC cell lines (AMC-HN3). The cell lines were cultured in DMEM (Invitrogen, Grand Island, NY) supplemented with 10% fetal bovine serum (Invitrogen) and 100 μg/ml penicillin/streptomycin and were incubated at 37 °C in a humidified incubator with an atmosphere of 5% CO_2. HNC cells were grown to approximately 50% confluence in vented 75-cm^2 culture flasks and were irradiated with 6-MV photon beam generated using a linear accelerator (CLINAC 600; Varian, Palo Alto, CA) at a dose of 2 or 4 Gy. The cell lines were maintained in a continuous culture for < 10 passages and were examined by performing PCR and FACS analyses before determining the expression of relevant proteins. By using the previously established human laryngeal squamous cell carcinoma cell line HN3 [18], clinically relevant fractionated radiation doses (2-Gy) at 2 days intervals were successively delivered. In addition, we established an isogenic model of successively irradiated HN3R cells by using a cumulative dose of 70 Gy. This model was designed to investigate radioresistance, which uses cells of the same origin that differ only in terms of radiosensitivity [19–21]. Moreover, we used radiosensitive HN30 cells and radioresistant HN31 cells [22].

Western blotting analysis

Protein extracts of AMC-HN3, AMC-HN3R, HN30, and HN31 cells treated with different reagents and incubated for different time points (0, 0.5, 1, 3, and 6 h) were resolved on a 7.5% polyacrylamide gel and were transferred to Hybond™ polyvinyl difluoride membranes (Amersham International Plc., Little Chalfont Buckinghamshire, UK). The membranes were blocked with 5% skimmed milk (Difco Laboratory, Detroit, MI, USA) for 1 h at room temperature, washed with PBS, and incubated overnight at 4 °C with primary antibodies. Antibodies against phosphorylated PI3K p85 (Tyr458)/p55(Tyr199), p85, Akt, phosphorylated Akt (Ser473), phosphorylated Akt (Thr308), Src, and phosphorylated Src (Tyr416) were purchased from Cell signaling Technology (Beverly, MA, USA). Antibody against PI3K p110α was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and that against β-actin was purchased from Sigma-Aldrich (St. Louis, Mo, USA). The antibodies were diluted in 5% bovine serum albumin in PBS. Next, the membranes were washed with Tween 20-containing PBS (PBST) and were incubated with anti-mouse horseradish peroxidase-conjugated IgG secondary antibody (dilution, 1:2000; Zymed, San Francisco, CA, USA) in PBST for 1 h. Specific binding was detected using an ECL kit (Amersham International Plc., Little Chalfont, Buckinghamshire, UK), according to the manufacturer's protocol. After the final washing, the membranes were developed using Supersignal West Pico Chemiluminescent substrates (Pierce Biotechnology, Rockford, IL, USA).

Reagents and cell viability assay

Anti-proliferative effects of HNC cell lines were determined by performing 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The cells (5000 cells/well) were incubated with indicated concentration of Ly294002 (Cayman Chemical, Ann Arbor, MI, USA), BYL719 (ApexBio, Houston, TX, USA), and dasatinib (Selleckchem, Houston, TX, USA) in a 96-well plate at 37 °C for 24 h. Next, 0.020 mL MTT solution (5 mg/mL in PBS) was added to each well. After incubation for 2 h at 37 °C, the medium was removed, 0.050 mL DMSO was added to each well, and the cells were incubated for 30 min at 37 °C to solubilize formazan crystals. OD at 570 nm was measured using a 96-well multi-scanner autoreader, and DMSO was used as a control. All experiments were performed in triplicate.

Clonogenic assay

Cancer cells were plated in a 6-cm dish at a density of 100, 500, 1000, and 4000 cells per dish. Briefly, the cells were treated with different does of the reagents and were plated in duplicate in the 6-cm dish. After 14 days, colonies formed were fixed with methanol and were stained with crystal violet. Colonies containing > 50 cells were counted under a microscope. Surviving fraction was calculated using the following formula: number of colonies/(cells seeded × plating efficiency). Clinically relevant fractionated radiation doses (2-Gy) at 2 days intervals were delivered and we established an isogenic model of irradiated HN3R cells by using a cumulative dose of 70 Gy.

RNA interference

HN31 cells were plated in 6-well plates (density, 1×10^5 cells/well) at 24 h before transfection. Next, the cells were transfected with 30 nmol siRNA against the gene encoding PI3K p85α (SC-36,217) by using an siRNA transfection reagent (Santa Cruz Biotechnology) and were grown for 72 h before performing western blotting analysis.

Xenograft model

To determine the antitumoral activity of combination treatment with PI3K inhibitor LY294002 and Src inhibitor dasatinib, we established a xenograft mouse model by subcutaneously injecting 5×10^6 HN31 cells into 5-to 6-week-old male athymic nude mice (*nu/nu*; Harlan-Sprague-Dawley). When tumor volume reached 50 mm^3, the mice were divided into the following four treatment groups, with six mice per group: (a) control, (b) 100 mg/kg LY294002 alone intraperitoneally (i.p.) daily for 7 days, (c) 100 mg/kg dasatinib alone i.p. daily for 7 days, and (d) 100 mg/kg LY294002 + dasatinib. All the mice were treated for 1 week and were sacrificed after approximately 4 weeks of treatment. Tumor volumes (V = l ∗ wˆ2 ∗ 0.5) and body weights of the mice were determined during the study period.