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Signaling pathways in adenoid cystic cancers:

Implications for treatment

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Abstract

Adenoid cystic cancers (ACC) in the head and neck are rare yet present a clinical dilemma. Although 5-y survivals are excellent, they have a propensity for late recurrences. Most of these cancers are initially treated with surgery followed by radiation. When recurrences happen, treatment options are limited both by the morbidity and low efficacy of re-irradiation and repeated surgical resection. Reported response rates to chemotherapy are low and targeted therapies may be one option. We, therefore, investigated signaling pathways that may be active in adenoid cystic cancers. Tissues from the last nine ACC patients resected at the University of Iowa were immunohistochemically stained with antibodies for EGFR, phosphorylated (P) Akt, and P-MAPK in order to molecularly characterize these tumors. An ACC cell line (ACC3) was also characterized by western blot. We found that seven of the nine tumor samples had strong expression of P-Akt and 5/9 had P-MAPK. None of them had EGFR expression. In the ACC3 cell line, similar data was found in that there was P-Akt and P-MAPK but no EGFR expression. We tested the HIV protease inhibitor nelfinavir (NFV) which has been shown to inhibit Akt signaling to see its effect on ACC3 cells. Both P-Akt and P-MAPK were inhibited with NFV in ACC3 cells and this resulted in growth inhibition and clonogenic death. In patients where re-irradiation or further surgery is not an option, a trial of NFV may be warranted.

Keywords

adenoid cystic; Akt; MAPK; EGFR; nelfinavir; signaling

Introduction

Adenoid cystic carcinomas (ACC) are rare tumors that account for about 1% of all head and neck cancers.¹ Although ACC may occasionally demonstrate rapid growth and metastases, it more commonly behaves in an indolent fashion. Despite this slow growth, local control with surgery is difficult to achieve presumably because of the extensive perineural extension that characterizes the tumor. Additionally, distant metastases are common and may be detected at the initial evaluation or many years after the primary tumor has been controlled.² The median/mean age at presentation is 47–56.^{3–5} Although 5-y disease-free survival (DFS) is in

the 65–70% range, the 15-y DFS drops to 30–40%.^{3–6} If followed long enough, 35% of patients will eventually develop metastatic disease.^{6,7}

The most common treatment of ACC is surgery followed by post-operative radiotherapy.^{3,4} When ACC recurs, management options are often limited both by the morbidity and low efficacy of re-irradiation and repeated surgical resection. Reported response rates to chemotherapy are low and when it occurs, the duration of the response is short lived.⁸

In an effort to explore possible targeted therapies for patients with recurrent ACC, we evaluated ACC tissue for expression of signaling proteins (EGFR, Akt and MAPK) that may permit the development of targeted therapy to inhibit tumor growth. We also evaluated these same proteins in vitro and evaluated the effect of the HIV protease inhibitor nelfinavir (NFV)—an inhibitor of Akt signaling—on ACC growth.

Results

Stored samples of paraffinized adenoid cystic cancers were stained for EGFR, phosphorylated (P) Akt and P-MAPK. They were interpreted by a single pathologist (R.A.R.) and both the intensity and quantity of staining was scored. Intensity was graded as: 1+ weak positivity with focal staining, 2+ clearly positive and homogenous staining and 3+ intense and homogenous immunostaining. Quantity was defined as no cells staining (0), <25% of the cells staining (1), 25–49% of the cells staining (2), 50–74% of the cells staining (3), >75% of the cells staining (4). As seen in Table 1, 7/9 samples had 2+ or 3+ staining of P-Akt with greater than 50% of the tumor being positive. All of the tumors had some P-Akt. Five of the nine were 3+ positive for P-MAPK. Figure 1 shows a representative tumor (sample 8) stained for P-Akt and P-MAPK. None of the tumors had any expression of EGFR. The reliability of this assay was confirmed by the positive control of hair follicles staining positive for EGFR.

An ACC cell line, ACC3 was obtained. On western blot analysis, it had the characteristics that we had seen in the tumors in that it was positive for P-Akt and P-MAPK but there was no expression of EGFR (Fig. 2). We have previously shown that the HIV protease inhibitor nelfinavir (NFV) can block Akt signaling.⁹ We were interested in seeing what happens to ACC3 cells when exposed to NFV. In Figure 3, we can see that NFV treatment at 8 μ M does result in downregulation of signaling through both the Akt and MAPK pathways. The dose of 8 μ M is physiologically relevant in that the serum concentration in patients taking NFV is 7–10 μ M.¹⁰ This dose of NFV also resulted in growth retardation of the cells (Fig. 4). The doubling time of ACC3 cells is 29 h and this increased to 87 h with NFV. We additionally tested the ability of these cells to form colonies (Fig. 5). Cells treated with NFV for 3 d had a greater than 40% reduction in their clonogenic surviving fraction.

Discussion

Adenoid cystic cancers (ACC) are slow growing tumors that locally result in significant morbidity due to their location (often adjacent to the skull base) and propensity to track along nerves. In addition they frequently metastasize and would benefit from systemic therapy. Standard chemotherapy has a poor track record of response. Patients with recurrent adenoid cystic cancers, therefore, have few therapeutic options. Targeted therapies are under development for cancers of the head and neck. There is, however, limited information available on signaling pathways that might be activated in adenoid cystic cancers. In an effort to elucidate the mechanisms that are involved in growth of ACC, we immunohistochemically stained nine tumors for phosphorylated or activated EGFR, Akt and

MAPK. Unexpected, none of the tumors demonstrated EGFR expression whereas seven showed P-Akt and five had P-MAPK expression.

There are currently no clinically applicable inhibitors of Akt or MAPK that are available. We and others have noted that some HIV protease inhibitors block Akt signaling.^{9,11} We tested the HIV protease inhibitor nelfinavir (NFV) and found that at clinically relevant doses, we could inhibit both Akt and MAPK signaling in the ACC3 cell line resulting in growth delay and decreased clonogenicity.

Recent trials of targeted therapies in advanced ACC have reported variable results. A number of agents have been tested. These include imatinib^{12–15} (which inhibits the c-KIT tyrosine kinase), gefitinib¹⁶ (which inhibits the EGFR tyrosine kinase), and trastuzumab¹⁷ (inhibits HER2/neu also known as EGFR2). It was recently shown that expression of HER2 in adenoid cystic cancers is rare at about 4%¹⁸ so it is not surprising that trastuzumab had little impact on disease. Most of these studies resulted in stabilization of disease but this could merely represent the indolent, slow growing nature of ACC. Princess Margaret Hospital recently did a Phase 2 study on the dual EGFR and HER2 inhibitor lapatinib in progressive ACC.¹⁹ They also reported stabilization of disease but since all their patients had to have documented disease progression, their time to progression of 3.5 mo may be indicative of some response.

ACC cell lines have not been successfully created in the US and that may be because of their slow growth rate. There are only a few ACC cell lines available and they were created in China and Japan. There is some confusion as to whether the ACC cell lines are actually from adenoid cystic cancers and it has been suggested that ACC3 may be HELA.²⁰ Due to the indolent growth of adenoid cystic cancers, any cell line that is created with measurable growth over a period of weeks would not likely represent the true biology of the disease. There is currently no other model system to study adenoid cystic cancer in vitro. However, the similarities in expression of P-Akt, P-MAPK and EGFR in the cell line we employed (ACC3) may assign clinical relevance to the in vitro study we performed.

Nelfinavir resulted in growth delay and decreased clonogenicity in ACC3 cells and downregulated signaling through Akt and MAPK which are both highly expressed in adenoid cystic cancers. Akt and MAPK are thought to be down-stream from EGFR. Phase I/II studies have shown little efficacy with inhibitors of up-stream signaling pathways. Nelfinavir has been in clinical use in HIV patients since 1997 and has well documented side effects.¹⁰ In patients with adenoid cystic cancers that cannot receive further surgery and radiation and have not responded to chemotherapy, a trial of nelfinavir may be warranted.

Methods

Immunohistochemistry

Institutional review board approval was obtained for acquiring paraffin embedded 5 micron sections from patients who were diagnosed with adenoid cystic cancers (ACC) to test for expressions of total EGFR (clone H11 from DAKO Corp.), and IHC-specific phosphorylated Ser 473 Akt and phosphorylated p44/42 MAPK antibodies (Cell Signaling Technologies).

Pretreatment with citrate buffer (pH: 6) in Microwave (1,000 W) twice (4 min, 3 min) to unmask, followed by topping in citrate buffer, followed by cooling to room temperature before washing twice in ddH₂O; Proteinase K digestion for 5 min followed by three washes in 1x PBS; antibody incubation of primary antibody, overnight at 4°C. Post incubation several washes in 1x PBS followed by incubation of the secondary (anti mouse) antibody,

followed by several washes for staining with the DAB immuno-staining system from the DAKO company.

Cells and nelfinavir

ACC-3 cells were kindly provided by Dr. Frank Ondrey from the University of Minnesota. They were cultured in high glucose DMEM (GIBCO®) plus 10% fetal calf serum (Hyclone) and 1% Penicillin-Streptomycin (GIBCO®/Invitrogen Corporation) and incubated at 37°C in humidified 5% CO₂/95% air (NuAIRE™).

The HIV protease inhibitor nelfinavir (NFV) (Pfizer company) was purchased from the University of Iowa Hospital and Clinics, department of Pharmacy in solid caplet form. They were crushed into powder, weighed and dissolved in 100% DMSO immediately prior to use. ACC3 cells were incubated with 8 µM (final concentration) NFV for 4 d prior to experiments.

Western blot

Harvesting cells was accomplished without trypsinization by rinsing plates with 1x PBS (GIBCO®), followed by scraping the cells off the plate with 50–100 µL Tris based lysis buffer (pH: 6.8) containing 2% SDS, 10% Glycerol, 1 mM sodium vanadate (Na₃VO₄) from Fisher Scientific, 5 mM sodium pyrophosphate from Sigma/Aldrich Company, 100 mM sodium fluoride (Naf) from Sigma/Aldrich Company as phosphatase inhibitors. 100 µL portions of dithio-threitol (DTT) Research Products International were stored at –20°C and mixed 1:20 (lysis buffer:DTT) immediately before use. Cells were boiled for 20 min, and then analyzed for protein concentration with the Lowry assay. Western blotting was performed by loading 50 µg protein/well onto a NuPAGE® 4–12% Bis-tris Gel 1.5 mm × 10 well (Invitrogen company) followed by blotting to a nitrocellulose membrane (Trans-blot® from the Bio-Rad company). After blotting the membrane was blocked in 1x PBS/1% Tween 20/5% powdered milk for 1 h and incubated overnight in primary polyclonal antibody such as EGFR[Tyr1173] from Millipore (Upstate), Akt[ser473], Akt[thr308], total AKT, P44/42 MAPK[thr202/tyr204] from the Cell Signaling company; and total MAPK-2 antibody from the SantaCruz Biotechnology, Inc. The Loading control was accomplished with GAPDH from the Millipore (Chemicon) Company. For detection of primary antibodies we used a sec. rabbit antibody (Cell Signaling); as well as sec. mouse antibody (from the Millipore company) for GAPDH. The binding detection was accomplished with ECL™ Amersham Hyperfilm, as developer we used Amersham ECL™ western blotting detection reagents. Images were digitalized using HP scanjet 7400c scanner and assembled with Microsoft power point. Quantitative analysis of westerns was performed with ImageJ software program from the NIH.

Cell growth assay

ACC-3 cells were removed from liquid nitrogen and incubated in DMEM (high Glucose) from the Gibco®, Invitrogen™, supplemented with 10% FCS (HyClone®, Thermo Scientific) and 1% Penicillin Streptomycin (Gibco®, Invitrogen™) at 37°C/21% O₂/4% CO₂. After three to four passes 50,000 cells were seeded into six-well plates (Costar®, Corning Inc.,) for each of the five time-points. Eight µM NFV was added at 0 time to all samples tested for drug interaction. Samples were harvested by trypsinization and counted after 0, 24, 48, 72 and 96 h and the N of 6 from each plate was averaged on a Microsoft Excel spreadsheet. The results were also calculated and plotted in MS-Excel.

Clonogenic assay

Two hundred cells per well, which were obtained from each time-point, were seeded into a six-well plate and incubated for 2 w under the same conditions as described above. Post-incubation the cells were stained with methylene blue. Colonies of 50 cells and above were counted and normalized to the control, graphic and statistical analysis of the N = 6 were calculated and documented in MS-Excel.

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Figure 1. Immunohistochemistry of a adenoid cystic cancer for phosphorylated Akt and MAPK. This is tumor 8 from Table 1.

Figure 2.

Western blot of ACC3 cells. Two identical blots were done at the same time with six lanes of ACC3 protein lysate on each blot. One blot was cut into six lanes and probed with: 1- Total MAPK, 2-MAPK (p44/42, Thr202/Tyr204), 3-Total Akt, 4-Akt (Ser473), 5-Akt (Thr 308) and 6-EGFR (Tyr1173). The other blot was probed for GAPDH as a loading control.

Figure 3. Protein lysates from ACC3 control (C) and nelfinavir (NFV, 8 μ M for 4 d) treated cells were run on a western and the blots probed with Akt (Ser473) and MAPK (p44/42, Thr202/Tyr204) antibodies. GAPDH was done as a loading control.

Figure 4. Cell growth curves of ACC3 cells treated with 8 μ M nelfinavir and control (treated with equal volume of the carrier DMSO). There was a minimum of three different samples for each time point on the graph.

Figure 5. Clonogenic survival of ACC3 cells with and without 8 μ M nelfinavir. Cells in multiple dishes were treated with NFV or equal volume carrier at time 0. Every 24 h a dish in each group was trypsinized and 200 cells plated in six separate dishes. These were allowed to grow for 14 d and colonies were then counted. The control counts were normalized to a value of 1.

Table 1

IHC of adenoid cystic cancers in nine tumor specimens

Sample #	Akt intensity (0-3)	Akt quantity (0-4)	MAPK intensity (0-3)	MAPK quantity (0-4)	EGFR (0-3)
1	1	3	0	0	0
2	2	4	0	0	0
3	1	3	0	0	0
4	3	4	1	3	0
5	3	4	3	3	0
6	2	4	3	1	0
7	3	4	3	3	0
8	3	3	3	1	0
9	3	4	3	2	0