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A Novel NHE1-Centered Signaling Cassette Drives Epidermal Growth Factor Receptor–Dependent Pancreatic Tumor Metastasis and Is a Target for Combination Therapy[1]

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Abstract

Pancreatic ductal adenocarcinoma (PDAC) is one of the most lethal cancers principally because of early invasion and metastasis. The epidermal growth factor receptor (EGFR) is essential for PDAC development even in the presence of Kras, but its inhibition with erlotinib gives only a modest clinical response, making the discovery of novel EGFR targets of critical interest. Here, we revealed by mining a human pancreatic gene expression database that the metastasis promoter Na^+/H^+ exchanger (NHE1) associates with the EGFR in PDAC. In human PDAC cell lines, we confirmed that NHE1 drives both basal and EGF-stimulated three-dimensional growth and early invasion via invadopodial extracellular matrix digestion. EGF promoted the complexing of EGFR with NHE1 via the scaffolding protein Na+/H+ exchanger regulatory factor 1, engaging EGFR in a negative transregulatory loop that controls the extent and duration of EGFR oncogenic signaling and stimulates NHE1. The specificity of NHE1 for growth or invasion depends on the segregation of the transient EGFR/Na+/H+ exchanger regulatory factor 1/NHE1 signaling complex into dimeric subcomplexes in different lipid raftlike membrane domains. This signaling complex was also found in tumors developed in orthotopic mice. Importantly, the specific NHE1 inhibitor cariporide reduced both three-dimensional growth and invasion independently of PDAC subtype and synergistically sensitized these behaviors to low doses of erlotinib.

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Introduction

Pancreatic ductal adenocarcinoma (PDAC) is one of the most deadly cancers, having a 5-year survival rate less than 5%, as even patients with apparently localized resectable tumors have occult distant micrometastases at the time of surgery [1]. Indeed, PDAC cell invasion occurs very early in the disease maybe even before the formation of an identifiable primary tumor [2]. Therefore, although surgery remains the cornerstone of cure, the need for adjuvant treatment modalities is of critical importance [3].

Abbreviations: EGFR, epidermal growth factor receptor; NHE1, Na+/H+ exchanger isoform 1; NHERF1, Na+/H+ exchanger regulatory factor 1; PDAC, pancreatic ductual adenocarcinoma

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Growth factors, in particular epidermal growth factor (EGF), are important mediators of desmoplastic stroma production [4], PDAC invasive growth [5], and resistance to apoptosis [6]. Accordingly, overexpression or active mutants of the EGF receptor (EGFR) correlate with distant metastases, resistance to chemotherapeutics, and decreased patient survival [7]. Indeed, in both PDAC tumors and cell lines, the EGFR is the principal activated receptor [8]. Furthermore, PDAC tumors and cell lines can be divided into "classical" and "quasimesenchymal" (QM-PDAC) subtypes, with the QM-PDAC subtype having far worse survival rates [9] and being less dependent on Kras but responsive to anti-EGFR treatment with the EGFR tyrosine kinase inhibitor erlotinib (OSI-774, Tarceva) [9]. This implies that PDAC cells, differently from other cancer types also dependent on mutant KRAS, still use the EGFR [10–12].

Accordingly, the current FDA-approved therapy for the first-line treatment of patients with locally advanced or metastatic PDAC combines chemotherapy with erlotinib but with limited survival benefits [13]. This small survival advantage, however, clearly points to the need for further research to identify agents that will significantly boost erlotinib's treatment efficacy. The interaction of EGFR with the multiple signaling nodes that regulate the different hallmarks of metastatic progression suggests that a combination of an EGFR inhibitor and other molecular targeted agents may offer an efficient approach to controlling PDAC metastasis and/or local invasion. In this respect, important strategies for identifying these agents are to determine 1) the key proteins involved in regulating EGFR expression and functional dynamics and 2) the principal downstream effectors of its function.

The scaffolding protein Na+/H+ exchanger regulatory factor 1 (NHERF1; SLC9A3R) regulates both EGFR trafficking and expression in breast [14] and biliary cancer [15]. The Na^+/H^+ exchanger isoform 1 (NHE1; SLC9A1), one of the principal drivers of metastasis, is an important downstream effector of EGFR-driven progression [16]. Furthermore, in breast cancer cells, the inhibition of the NHE1 during the administration of various chemotherapeutic drugs [17] synergistically potentiates their antineoplastic effects, supporting the hypothesis that a combination therapy targeting both NHE1 and EGFR in PDAC may increase their individual antitumor activity. Although PDAC cell lines express NHE1, its role and dynamics in transducing the EGFR neoplastic signal in PDAC are still unknown.

We demonstrate that in PDAC exists a novel prometastatic protein-protein signaling complex centered around EGFR, NHERF1, and NHE1. The EGFR and NHERF1 are engaged in a proteasome-dependent, reciprocal feedback regulatory loop in which NHERF1 and EGFR interact to regulate their expression levels and functions. This provides a stimulatory signal for NHE1 activity, which promotes increased invadopodia proteolytic activity, enhanced local invasion, and three-dimensional (3D) growth of multicellular tumor spheroids. In line with this, subthreshold concentrations of the NHE1 inhibitor cariporide sensitized the cells to erlotinib, determining a synergistic block of 3D colony growth and invasive capacity. Altogether, these data demonstrate the need to repurpose inhibitors of NHE1, such as cariporide, for use in the oncological context and especially in treatment of PDAC [18].

Materials and Methods

Cell Culture

Experiments were performed on well-established human pancreatic cancer cell lines: PANC-1, BXPC3, MiaPaCa-2, and CAPAN-2. All cells were kept at 37°C in humidified air containing 5% CO_2. Panc-1 cells were grown in bicarbonate-buffered Dulbecco's minimal essential medium (pH 7.4). All other cells were cultured in bicarbonate-buffered RPMI 1640 medium (pH 7.4). Media were always supplemented with 10% fetal calf serum.

3D growth, In Vitro Invasion, Invadopodial Extracellular Matrix (ECM) Proteolysis, and Migration Assays

The details of the methods for these assays for each cell line are described in the Supplemental Methods.

Immunofluorescence, Coimmunoprecipitation, and Immunoblot Analysis

Interactions of NHERF1 with EGFR and NHE1 were analyzed in PANC-1 cells transiently transfected with WT-NHERF1 or the respective empty vector and stimulated or not with EGF for the indicated times. These assays were then performed as described in Supplemental Methods.

Orthotopic Implantation of Human Pancreatic Tumor Cell Lines and Immunohistofluorescent Staining of Surgical Specimens

All experiment were performed in severe combined immunodeficient mice, strain C.B-17/Ztm-scid of both sexes or nude mice, strain NMRI-Fox1 nu/nu and were performed according to protocols approved by the institutional animal use committee and in accordance with the Declaration of Helsinki protocols. Detailed protocols can be found in Supplementary Methods.

Data Mining from Gene Expression Data

The potential contribution of NHE1 in EGFR function in PDAC was explored using the Exploratory Gene Association Networks (EGAN) program with a Microarray U133 A/B Affymetrix GeneChip data set derived from mRNA extracted from patients who had undergone pancreatic surgery in the University Hospitals of Kiel and Dresden, Germany, and from a series of pancreatic tumor, normal, and stellate cell lines [19]. Full details are described in Supplementary Methods.

Statistical Procedures

Data correspond to at least three independent experiments, each of which was done in triplicate. Results are presented as means ± standard error. The data for each condition were subject to analysis of variance followed by Dunnet *post hoc* test when comparing three or more conditions or evaluated using Student's *t* test when comparing only two conditions. Significant differences were considered with values of $P < .05$. The results of single and combined treatments with erlotinib and cariporide on 3D growth were analyzed according to published methods [20] and are described in more detail in Supplemental Methods.

Results

NHE1 Is Associated with EGFR in PDAC and Is a Major EGFR-Driven pH_i Regulator

To explore the potential contribution of NHE1 in EGFR function in PDAC, we first used the EGAN (UCSF) program to interrogate a Microarray U133 A/B Affymetrix GeneChip database derived from mRNA extracted from microdissected patient tissues including pancreatic tumor and normal epithelium, stromal tissue, and stromal chronic pancreatitis specimens and from a set of pancreatic tumor, normal, and stellate cell lines [19]. As shown in the EGAN-produced interactome map (Figure 1*A*), data mining of a normal and PDAC