Cellular Signalling 27 (2015) 373–381

Contents lists available at ScienceDirect

Cellular Signalling

journal homepage: www.elsevier.com/locate/cellsig

ErbB2 signaling activates the Hedgehog pathway via PI3K–Akt in human esophageal adenocarcinoma: Identification of novel targets for concerted therapy concepts

Maxim Kebenko [a], Astrid Drenckhan [b], Stephanie J. Gros [b], Manfred Jücker [c], Nicole Grabinski [c], Florian Ewald [c], Astrid Grottke [c], Alexander Schultze [a], Jakob R. Izbicki [b], Carsten Bokemeyer [a], Jasmin Wellbrock [a,1], Walter Fiedler [a,*,1]

[a] Hubertus Wald Tumorzentrum, Department of Oncology–Hematology, Bone Marrow Transplantation and Pneumology, University Cancer Center, Hamburg, Germany
[b] Department of General, Visceral and Thoracic Surgery, Hamburg, Germany
[c] Institute of Biochemistry and Signal Transduction, University Medical Center Eppendorf, Hamburg, Germany

ARTICLE INFO

Article history:
Received 21 October 2014
Accepted 21 November 2014
Available online 27 November 2014

Keywords:
Esophageal cancer
Hedgehog
Gli
Non-canonical
ErbB2–Akt

ABSTRACT

The Hedgehog pathway plays an important role in the pathogenesis of several tumor types, including esophageal cancer. In our study, we show an expression of the ligand Indian hedgehog (Ihh) and its downstream mediator Gli-1 in primary resected adenocarcinoma tissue by immunohistochemistry and quantitative PCR in fifty percent of the cases, while matching healthy esophagus mucosa was negative for both proteins. Moreover, a functionally important regulation of Gli-1 by ErbB2–PI3K–mTORC signaling as well as a Gli-1-dependent regulation of Ihh in the ErbB2 amplified esophageal adenocarcinoma cell line OE19 was observed. Treatment of OE19 cells with the Her2 antibody trastuzumab, the PI3K–mTORC1 inhibitor NVP BEZ235 (BEZ235) or the knockdown of Akt1 resulted in a downregulation of Gli-1 and Ihh as well as in a reduction of viable OE19 cells *in vitro*. Interestingly, the Hedgehog receptor Smo, which acts upstream of Gli-1, was not expressed in OE19 cells and in the majority of primary human esophageal adenocarcinoma, suggesting a non-canonical upregulation of Gli-1 expression by the ErbB2–PI3K axis. To translate our findings into a therapeutic concept, we targeted ErbB2–PI3K–mTORC1 by trastuzumab and BEZ235, combining both compounds with the Gli-1/2 inhibitor GANT61. The triple combination led to significantly stronger reduction of tumor cell viability than cisplatinum or each biological alone. Therefore, concomitant blockage of the ErbB2–PI3K pathway and the Hedgehog downstream mediator Gli-1 may provide a new therapeutic strategy for esophageal cancer.

1. Introduction

Esophageal adenocarcinoma represents a prevalent type of cancer worldwide, showing an increase of incidence in the last years. Frequently, it is associated with Barret-esophagus in patients with gastro-esophageal reflux. Fifteen to twenty-five percent of esophageal adenocarcinoma show an amplification of ErbB2, which belongs to the family of epidermal growth factor receptor (EGFR) and correlates with poor long-term survival [1]. In contrast to the EGFR subtypes ErbB1, ErbB3 and ErbB4, a ligand for ErbB2 has not been identified so far. However, ErbB2 forms homodimers with itself and heterodimers with other EGFR subtypes, which results in tyrosine phosphorylation of several receptors [2]. The most frequent downstream messengers of ErbB2 signaling are phosphoinositid-3-kinase (PI3K)–Akt (proteinkinase B), Src-STAT3 and RAS-ERK [3–5].

The PI3K–Akt pathway plays an important role in carcinogenesis, causing regulation of proliferation, survival and inhibition of apoptosis. The PI3K subclass IA consists of a regulatory (p85) and catalytic (p110) subunit [6]. The catalytic subunit phosphorylates phosphatidylinositol-4,5-trisphosphat (PIP_2) to phosphatidylinositol-3,4,5-trisphosphat (PIP_3), leading to phosphorylation of Akt with the consecutive upregulation of protein synthesis and proliferation by mTORC1/2 complex and GSK-3β, respectively [7,8]. PI3K–Akt signaling is aberrantly activated in different tumor entities, providing an important base for pharmacological targeting. Especially, inhibitors of the PI3K, Akt and mTORC as well as dual PI3K–mTORC inhibitors are currently investigated in several preclinical and clinical studies [9,10].

Since activation of the PI3K–Akt pathway has been shown to potentate Hedgehog–Gli activity through diverse mechanisms [11], we

* Corresponding author at: Hubertus Wald Tumorzentrum, Department of Oncology–Hematology, Bone Marrow Transplantation and Pneumology, University Cancer Center Hamburg, Martinistrasse 52, 20246 Hamburg, Germany. Tel.: +49 40 7410 53919; fax: +49 40 7410 52186.
E-mail address: fiedler@uke.de (W. Fiedler).
[1] Contributed equally.

http://dx.doi.org/10.1016/j.cellsig.2014.11.022
0898-6568/

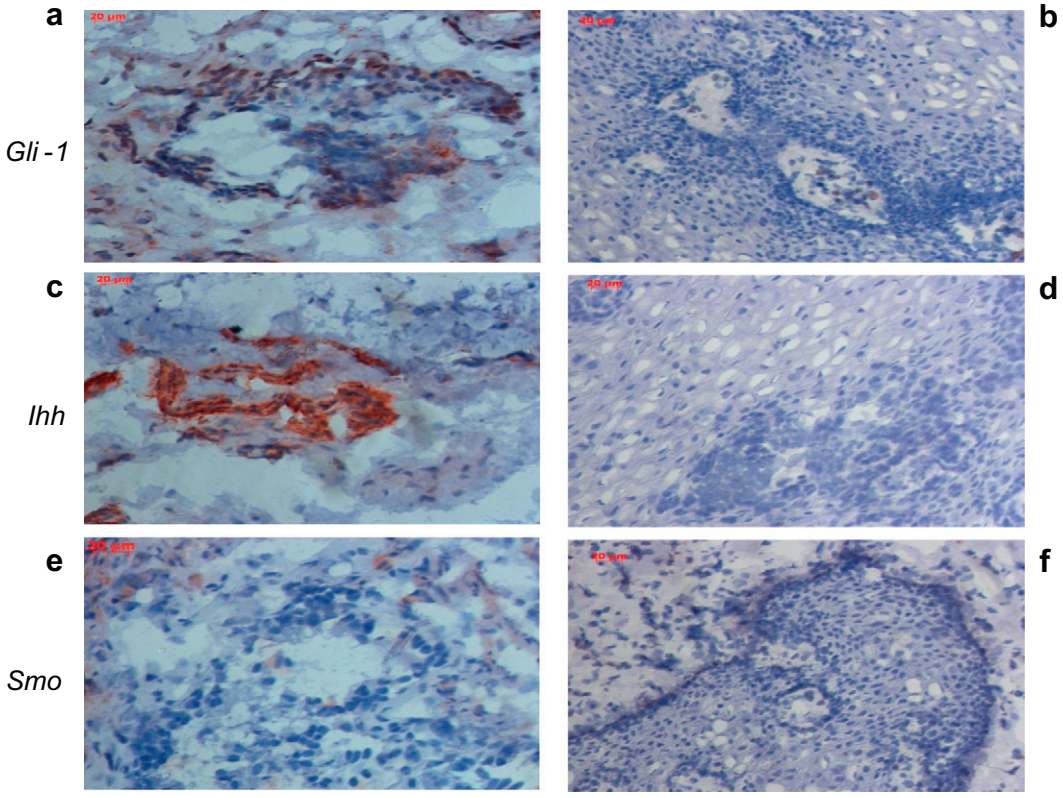

Fig. 1. Immunohistochemistry for Gli-1, Ihh and Smo of primary human cancer and adjacent normal esophageal tissue. Primary human esophagus tumor (a, c, e) and normal tissue sections (b, d, f) were stained for Gli-1 (a-b), Ihh (c-d) and Smo (e-f). Gli-1 and Ihh-expression was found in tumor cells, whereas normal tissue was negative for both. Smo-expression was expressed neither in tumor nor in normal tissue ($n = 10$).

focused our investigation on a possible interaction between both pathways.

The Hedgehog pathway is involved in the regulation of proliferation, self-renewal and chemotherapy resistance of human tumors such as medulloblastoma [12], basal cell carcinoma [13] or gastro-esophageal cancer [14]. The three ligands, Sonic (Shh), Indian (Ihh) and Desert (Dhh) hedgehog are glycoproteins with a distinct tissue-specificity [15–17]. Ligand-binding to the receptor PTCH, which acts as a membrane-associated inhibitor of smoothened receptor (Smo), causes the translocation of Smo to the primary cilium, activating the serine/threonine kinase STK36 and phosphorylating SUFU for nuclear stabilization of glioma-associated oncogene (Gli). Gli-1, Gli-2 and Gli-3 are transcriptional zink-finger co-factors, which induce transcriptional activation of Hedgehog target genes [18]. Mutations, leading to a loss of PTCH, or a gain of Smo function [19,20] as well as to an overexpression of Hedgehog ligands [21], are frequently detected in several types of cancer.

In the current project, we analyzed the effect of trastuzumab, a recombinant human monoclonal ErbB2-antibody, and BEZ235, a small molecule pan-PI3K–mTORC1 inhibitor, on the viability of an ErbB2-overexpressing, human esophageal adenocarcinoma cell line OE19. Special emphasis was put on the regulation of Hedgehog signaling by the ErbB2–PI3K–mTORC cascade. Secondly, samples of primary human esophageal cancer were explored for expression of members of the Hedgehog pathway. Furthermore, anti-tumor effects by combining inhibitors of the ErbB2–PI3K-axis with the Gli-1/2 inhibitor GANT61 were investigated.

2. Methods and materials

2.1. Cell culture

OE19 cells (human esophageal adenocarcinoma with the ErbB2 amplification) were grown in RPM1 Medium 1640 (1x) (Gibco, Carlsbad, California) supplemented with 10% FBS and 1% penicillin/streptomycin. HEK293 cells (Human Embryonic Kidney 293 cells) were grown in DMEM (Lonza, Basel, Switzerland) supplemented with 10% FBS.

2.2. Cell viability assay

Vital cells were counted either by the Vi-CELL™ XR (Beckman Coulter, Germany) or the standard trypan blue exclusion assay using a Neubauer chamber. The samples were incubated with the following compounds for 72 h: DMSO (control), trastuzumab at a dose range 20 μg/ml–200 μg/ml, BEZ235 0.01 μM–10 μM, INK128 0.01 μM–10 μM, GANT61 10 μM–90 μM, and cisplatinum 2.5 μg/ml–150 μg/ml as well as with individual combinations.

2.3. Apoptosis assay

For measuring apoptosis, either 5×10^6 cells were harvested by trypsinization and analyzed by Annexin-V FITC (BD Biosciences, San Jose, California) and propidium iodide (Sigma-Aldrich, St. Louis, Missouri) in flow cytometry using FACSCalibur and CellQuestPro software (BD Biosciences). The samples were incubated with DMSO (control) or 70 μM GANT61 for 6 h and 20 h.

2.4. Transformation and plasmid-DNA-preparation

200 μl competent cells (*Escherichia coli*, Invitrogen Life Technologies, Carlsbad, California) were incubated with 1 μg plasmid DNA (Sigma-Aldrich) following the manufacturer's instruction. The QIAGEN Plasmid Maxi Kit (Venlo, The Netherlands) was used to purify plasmid DNA.