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Co-expression of receptor tyrosine kinases in esophageal adenocarcinoma and squamous cell cancer

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Abstract. This study aimed to define the co-expression pattern of target receptor tyrosine kinases (RTKs) in human esophageal adenocarcinoma and squamous cell cancer. The co-expression pattern of vascular endothelial growth factor receptor (*VEGFR)1-3*, platelet-derived growth factor receptor (*PDGFR)α/ß* and epidermal growth factor receptor 1 (*EGFR1)* was analyzed by RT-PCR in 50 human esophageal cancers (35 adenocarcinomas and 15 squamous cell cancers). In addition, IHC staining was applied for the confirmation of the expression and analysis of RTK localisation. The adenocarcinoma samples revealed *VEGFR1* (97%), *VEGFR2* (94%), VEGFR3 (77%), *PDGFRα* (91%), *PDGFRß* (85%) and EGFR1 (97%) expression at different intensities. Ninety-four percent of the esophageal adenocarcinomas expressed at least four out of six RTKs. Similarly, squamous cell cancers revealed *VEGFR1* (100%), *VEGFR2* (100%), VEGFR3 (53%), *PDGFRα* (100%), *PDGFRß* (87%) and EGFR1 (100%) expression at different intensities. All esophageal squamous cell carcinomas expressed at least four out of six RTKs. While *VEGFR1-3* and *PDGFRα* and *EGFR1* was expressed by tumor cells, *PDGFRß* was restricted to stromal cells, which also depicted a *PDGFRα* expression. Our results revealed a high rate of RTK co-expression in esophageal adenocarcinoma and squamous cell cancer and may encourage application of multi-target RTK inhibitors within a multimodal concept as a promising novel approach for innovative treatment strategies.

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Abbreviations: EADC, esophageal adenocarcinoma; ESCC, esophageal squamous cell cancer; RTK, receptor tyrosine kinase; BP, base pair

Key words: vascular endothelial growth factor receptor, epidermal growth factor receptor, platelet-derived growth factor receptor, esophageal adenocarcinoma, esophageal squamous cell cancer

Introduction

Esophageal cancer is the sixth most common cause of cancer-related deaths worldwide. It is well known that the prognosis for esophageal cancer is worse than for other digestive cancers in spite of multimodality treatment, thus there is an urgent need to improve this situation. It is a highly aggressive malignancy with a propensity for invasive local growth, early lymphatic spread and vascular invasion. Radical surgery as the treatment of choice offers 5-year survival rates of only 30% (1). Advances in careful preoperative selection, extensive surgery with improved techniques and conventional (neo)adjuvant chemo- and radiotherapy have only shown a limited improvement of prognosis (2,3). The rising incidence of esophageal adenocarcinoma (EADC) and the dismal prognosis associated with current treatment strategies warrant a search for innovative therapies.

Receptor tyrosine kinases (RTKs) are transmembrane proteins containing extracellular ligand-binding domains and intracellular catalytic domains (4). Receptor binding of the respective ligand results in RTK autophosphorylation and a consecutive *Mek1/2* and *Erk1/2* activation via *Raf* or *Ras* (5,6). The expression of functional receptors and their respective ligands by tumor cells also raises the possibility of autocrine loops and is critical for autostimulation and progression (7,8).

Hitherto, growth factors such as vascular endothelial growth factor (*VEGF*) and platelet-derived growth factor (*PDGF*) and their receptors have been considered relevant in the process of angiogenesis and dissemination in esophageal cancer, whereas epidermal growth factor receptor (*EGF/EGFR*) was correlated with tumor growth and local invasion (9-11). As part of the tyrosine kinase family, *PDGF* receptors are involved in multiple tumor-associated processes, such as enhancing tumor angiogenesis by the recruitment and regulation of tumor fibroblasts and pericytes (12).

As new multi-target tyrosin kinase inhibitors are emerging and enriching the therapy in various malignancies, our aim was to define the expression pattern of target RTKs in human EADC and ESCC and thus give a rationale for a possible new therapeutic strategy (13,14).

Materials and methods

Tissue source and storage. Tumor samples were obtained from 50 consecutive patients undergoing elective surgery for esophageal cancer with curative intent [35 samples of adenocarcinoma (EADC; only adenocarcinoma of the esophagogastric junction type I), 15 samples of ESCC] at the Department of General and Abdominal Surgery, University of Mainz, between 2005 and 2006. Specimens were conventionally fixed in formalin for histopathological analysis. In addition to the conventional processing of tissues in formalin for standard analysis, small samples of each specimen were stored in cryovials, shock-frozen in liquid nitrogen immediately after extirpation and stored at -80˚C until further processing. These tumor tissues originated from the center of the tumor. As control tissues, samples of healthy esophageal mucosa 2 cm apart from the proximal tumor margin were collected from the same surgical resectate. Informed consent was obtained before the respective tissue was collected.

Immunohistochemistry. Five paraffin-embedded tissue samples of esophaegal mucosa, adenocarcinoma and squamous cell cancer, respectively, were generously provided by Dr S. Biesterfeld (Institute of Pathology, University of Mainz) and were screened for *VEGFR1-3*, *PDGFRα/β* and *EGFR1* protein expression by immunohistochemistry (Table I). The tissues were deparaffinized, rehydrated and subsequently incubated with the respective primary antibody (Table I). The secondary antibody (anti-rabbit-mouse-goat-antibody) was incubated for 15 min at room temperature, followed by incubation with strepavidin-POD (Dako, Germany) for 15 min. Antibody binding was visualized using AEC-solution (Dako). The tissues were then counterstained by haemalaun solution (Dako).

RNA isolation and reverse transcription-PCR. RNA isolation was performed using an RNeasy kit according to the manufacturer's recommendations (Qiagen, Hilden, Germany). The transcription of *ß-actin*, *VEGFR1*, *VEGFR2*, *VEGFR3*, *PDGFRα*, *PDGFRβ* and *EGFR1* was analyzed by a two-step RT-PCR: reverse transcription was performed with 2 μg of RNA (20 μl total volume; Omniscript RT kit, Qiagen) according to the recommendations of the manufacturer. In total, 0.5 μl of the cDNA (50 ng) was used as a template for the specific PCR reactions. Primers applied were *ß-actin* forward: 5'-TGA CGG GGT CAC CCA CAC TGT GCC CAT CTA-3' and reverse 5'-CTA GAA GCA TTT GCG GTG GAC GAC GGA GGG-3' [661 base pairs (bp) fragment], *VEGFR1* forward: 5'-TGG GAC AGT AGA AAG GGC TT-3' and reverse: 5'-GGT CCA CTC CTT ACA CGA CAA-3' (394 bp), *VEGFR2* forward: 5'-CAT CAC ATC CAC TGG TAT TGG-3' and reverse: 5'-GCC AAG CTT GTA CCA TGT GAG-3' (400 bp), *VEGFR3* forward: 5'-CCC ACG CAG ACA TCA AGA CG-3' and reverse: 5'-TGC AGA ACT CCA CGA TCA CC-3' (380 bp), *PDGFRα* forward: 5'-CTC CTG AGA GCA TCT TTG AC-3' and reverse 5'-AAG TGG AAG GAA CCC CTC GA-3', *PDGFRβ* forward: 5'-TCC TCA ATG TCT CCA GCA CCT TC-3' and reverse 5'-ACC ACA GTC TGC ACT GCG TTC-3' (547 bp) and *EGFR1* forward: 5'-TCT CAG CAA CAT GTC GAT GGA-3' and reverse: 5'CGC ACT TCT TAC ACT TGC GG-3' (474 bp). For amplification, a DNA Engine PTC200 (MJ Research, Watertown, MA) thermocycler was used. Cycling conditions of the respective PCRs were as follows: intitial denaturation (4 min at 94˚C) followed by the respective number of cycles (ß-*actin*, 28; *VEGFR1*, 36; *VEGFR2*, 38; *VEGFR3*, 38; *PDGFRα*, 38; *PDGFRβ*, 36 and *EGFR1*, 38) of denaturation (1 min at 94˚C), annealing (45 sec; ß-*actin*, 52˚C; *VEGFR1*, 60˚C; *VEGFR2*, 62˚C; *VEGFR3*, 62˚C; *PDGFRα*, 57˚C; *PDGFRβ*, 64˚C and *EGFR1*, 60˚C) and elongation (1 min at 72˚C). After the last cycle, a final extension (7 min at 72˚C) was added and thereafter the samples were kept at 4˚C. The product (7 μl) was run on a 1.8% agarose gel, stained by ethidium bromide and analyzed under UV light. The evaluation of the expression was performed semiquantitatively according to the following gardes: negative, 0; weak, 1; medium, 2 and strong, 3.

Results

Immunohistochemical staining of RTKs in normal esophageal mucosa and cancer samples. Negative controls of esophageal mucosa and of cancer specimens remained negative for all the samples.

RTK expression in normal esophageal mucosa varied from absent (*VEGFR1*, *VEGFR2*, *PDGFRβ*) to intermediate (*VEGFR3*, *PDGFRα*) and strong (*EGFR1*; Fig. 1A).

Cancer cells stained for *VEGFR1*, *VEGFR2*, *VEGFR3*, *PDGFRα* and *EGFR1*, but not for *PDGFRβ*, whereas stromal cells stained for *PDGFRα* and *PDGFRβ* (Fig. 1B). In cancer cells *VEGFR1*, *VEGFR2*, *VEGFR3* and *PDGFRα* revealed a predominantly cytoplasmic and lesser membranous localisation, whereas *EGFR1* revealed a similar cytoplasmic and membranous staining. Additional nuclear staining was observed for *VEGFR2* and *VEGFR3*. Stromal cells revealed a cytoplasmic *PDGFRα* and *PDGFRβ* localisation.

RTK expression patterns in esophageal adenocarcinoma. *VEGFR1*, *VEGFR2*, *VEGFR3*, *PDGFRα*, *PDGFRβ* and *EGFR1* expression in esophageal adenocarcinoma samples revealed varying transcription intensities. *VEGFR1* expression was observed in 97% (34/35) of the samples and varied from strong (37%) to intermediate (43%) and weak (17%; Fig. 2A). *VEGFR2* expression was found in 94% (33/35) of the esophageal adenocarcinoma specimens and ranged from weak (29%), to intermediate (20%) and strong (45%). The overall expression rate of *VEGFR3* was 77% (27/35) with a weak expression in 40%, an intermediate expression in 14% and a strong expression in 23%. *PDGFRα* expression was observed in 91% (32/35) of the samples. A strong *PDGFRα* expression was found in 11%, whereas 26% revealed an intermediate and 54% a weak expression. *PDGFRβ* expression was seen in 85% (30/35) and varied from weak (40%) to intermediate (14%) and strong (31%). The expression rate of *EGFR1* was

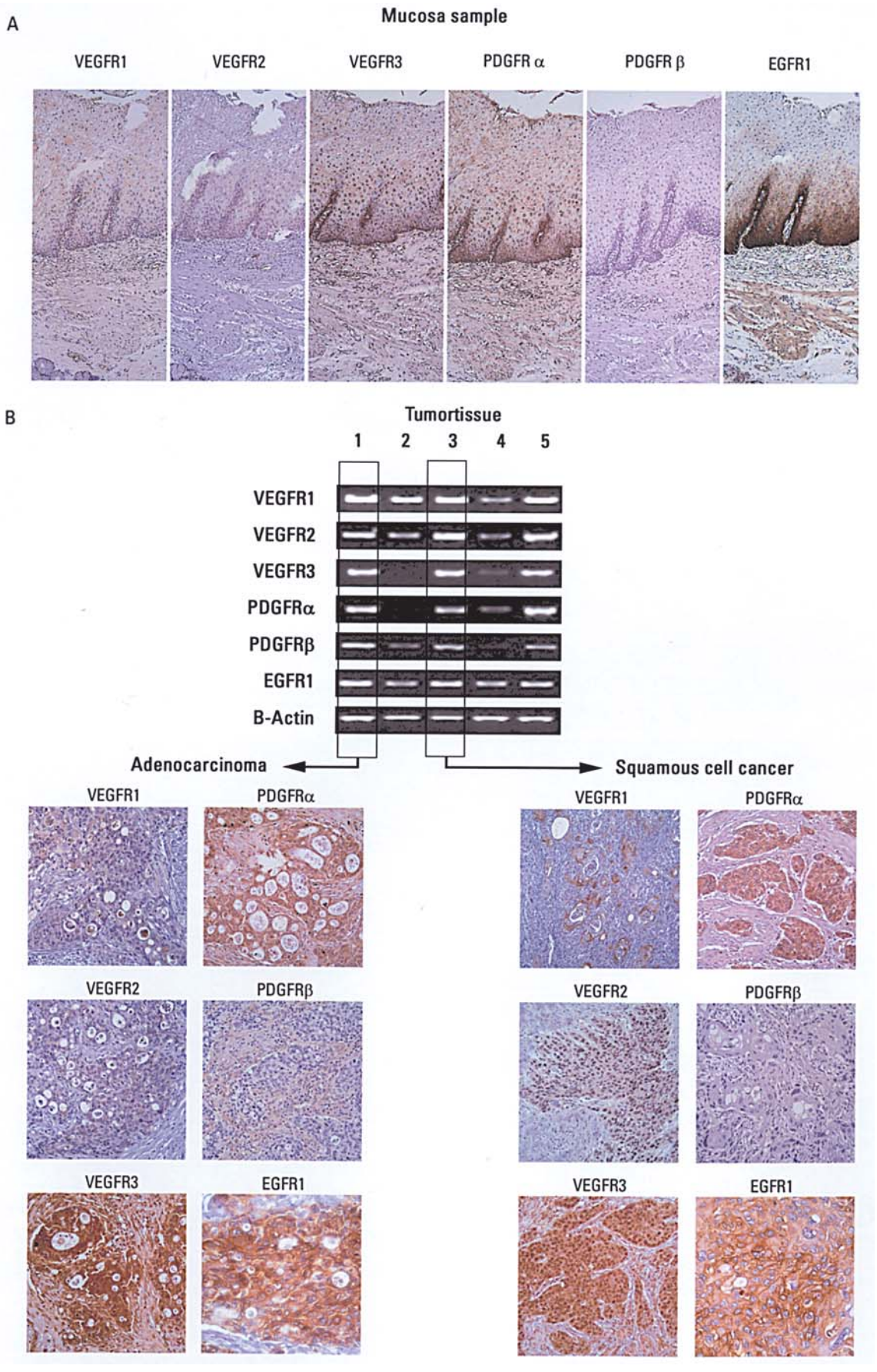

Figure 1. (A) The IHC staining of healthy esophageal mucosa for *VEGFR1-3*, *PDGFR-α/β* and *EGFR1*. RTK expression in healthy esophageal mucosa varied from absent (VEGFR1, VEGFR2, PDGFRß) to intermediate (*VEGFR3*, *PDGFRα*) and strong [EGFR1; (A)]. (B) The exemplary transcription profile of 10 esophageal cancers and immunohistochemical analyses of an adenocarcinoma and squamous cell cancer, respectively (B). Cancer cells stained for *VEGFR1*, *VEGFR2*, *VEGFR3*, *PDGFRα* and *EGFR1* but not for *PDGFRβ*, whereas stromal cells stained for *PDGFRα* and *PDGFRβ* (B). PCR detected RTK expression more sensitively than IHC.

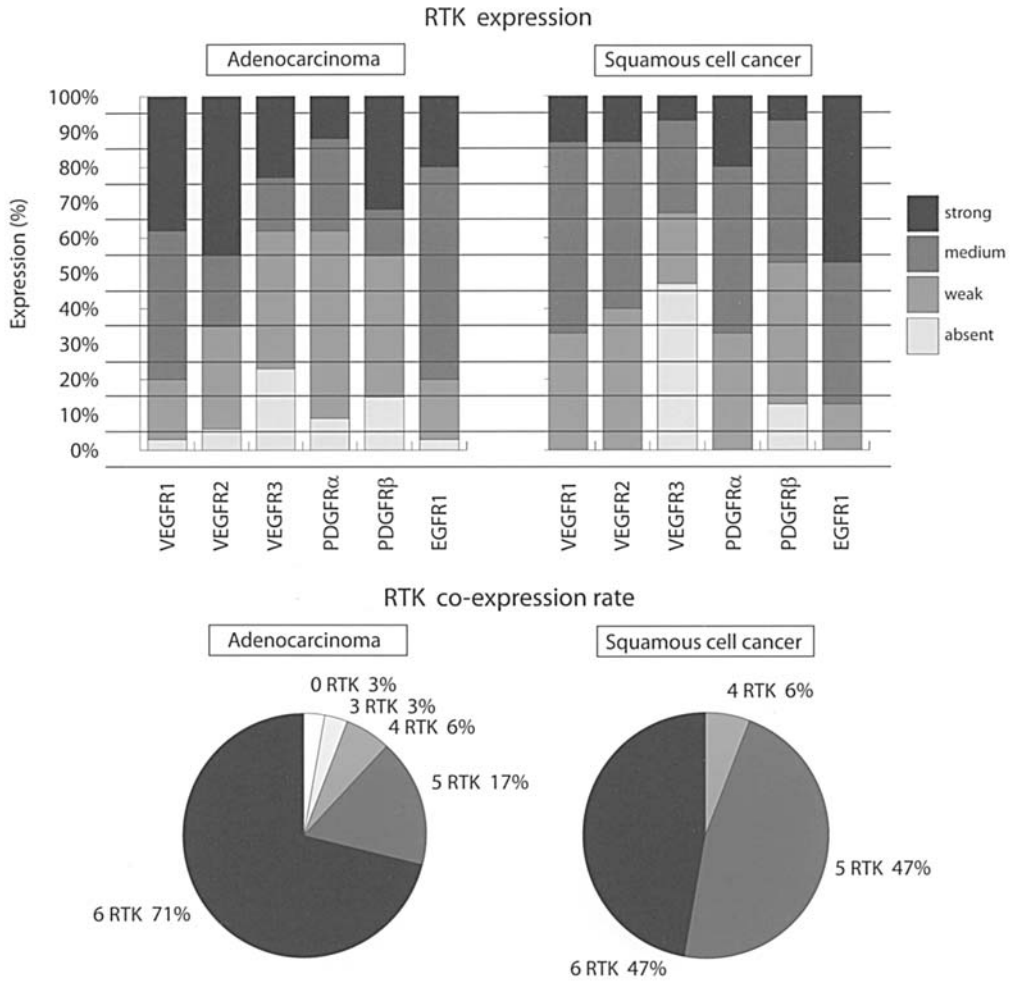

Figure 2. (A) The expression profile of RTKs *VEGFR1-3*, *PDGFRα/β* and *EGFR1* in human esophageal adenocarcinoma and squamous cell cancer. (B) The co-expression rates of *VEGFR1-3*, *PDGFRα/β* and *EGFR1* in human esophageal adenocarcinoma and squamous cell cancer.

Table I. Antibody characteristics.

Target	Antibody	Dilution	Incubation	Company
VEGFR1	Flt-1 (C-17)	1:100	4 h	Santa Cruz Biotechnology, CA, USA
VEGFR3	Flt-4 (C20)	1:200	2 h	Santa Cruz Biotechnology, CA, USA
PDGFRα	PDGFRα (C-20)	1:200	2 h	Santa Cruz Biotechnology, CA, USA
PDGFRß	PDGFRß (28 E1)	1:200	2 h	Cell Signalling Technology, MA, USA
EGFR1	AM207-5ME	1:1	1 h	BioGenex, CA, USA

97% (34/35) and varied from weak (17%), to intermediate (60%) and strong (20%). Samples (71%) revealed a co-expression of six receptors, 17% of five receptors, 6% of four receptors and only 6% showed a co-expression of three receptors or less (Fig. 2B).

RTK expression patterns in esophageal squamous cell carcinoma. VEGFR1, *VEGFR2*, *VEGFR3*, *PDGFRα*, *PDGFRβ* and *EGFR1* expression revealed varying transcription intensities in esophageal squamous cell cancer. Notably, *VEGFR1*, *VEGFR2*, *EGFR1* and *PDGFRα* expression was observed in the samples (100%) analysed. *VEGFR1* varied from strong (13%) to intermediate (54%) and weak (33%; Fig. 2A). *VEGFR2* expression ranged from weak (40%), to intermediate (47%) and strong (13%). The overall expression rate of *VEGFR3* was 53% (8/15) with a weak expression in 20%, an intermediate expression in 27% and a strong expression in 6%. A strong *PDGFRα* expression was found in 20%, whereas 47% revealed an intermediate and 33% a weak expression. *PDGFRβ* expression was seen in 87% and

om weak (40%) to intermediate (40%) and strong ; expression of *EGFR1* varied from weak (13%), to intermediate (40%) and strong (47%).

Forty-seven percent of the samples revealed a co-expression of six receptors, 47% of five receptors, 6% of four receptors, while no sample showed a co-expression of three receptors or less (Fig. 2B).

Discussion

This is the first study to analyze the co-expression profile of a series of RTKs in human EADC and ESCC. We performed this study in order to assess the possibility of a therapy with novel multi-targeted RTK inhibitors in esophageal cancer, as we assumed that tumors co-expressing multiple RTKs are functionally more dependent on ligand binding and more prone to deprivation of those stimuli. RTKs most frequently targeted by available small molecules were chosen for this analysis.

RTKs such as *VEGFR1-3*, *PDGFRα/β* and *EGFR1* undergo phosphorylation following ligand binding resulting in tyrosine kinase activity and concomitant activation of the *Ras-Raf-Mek1/2-Erk1/2* pathways (5,6). In the tumor stroma, *VEGFR1* and *VEGFR2* are expressed in endothelial cells, whereas *VEGFR3* is largely restricted to lymphatic endothelial cells and *PDGFRα/β* to pericytes. Many RTKs are also expressed by the tumor cells themselves. Depending on the location of the RTK in tumor cells, endothelial cells or pericytes, the consequences are tumor cell proliferation, dissemination or angiogenesis.

Several studies have analysed the impact of RTK expression on clinicopathological parameters in EADC and ESCC. In an earlier study, cytoplasmic *VEGFR1* expression of ESCC revealed a trend towards poorer nodal status, although it did not correlate with the prognosis (9). In contrast, cytoplasmic *VEGFR2* expression has also been localised in ESCC, but did not correlate with clinicopathological factors or prognosis (9). Furthermore, the expression of *VEGFR2* was previously observed in immature tumor blood vessels while *VEGFR3* was detected in lymphatic vessels in esophageal cancer (10,11). As expected, the expression of the VEGFR3 ligand, VEGFC, correlated with the depth of tumor invasion, tumor stage and lymph node metastasis in esophageal cancer (11). In another recent study, the expression of the ligands VEGFA, VEGFB, VEGFD and VEGFR3 correlated with the microvessel density and lymphatic dissemination (15). These data indicate the high relevance of VEGFR1-3 and their ligands for tumor progression. Our description of a high RTK expression rate supports these results.

The first *in vitro* analyses revealed that the expression rate of PDGFRß was significantly higher in tumor tissues than in para-tumoral and normal tissues (16). Matching these observations, imatinib induced apoptosis in PDGFRß-positive esophageal cancer (17). A PDGF-BB expression was observed in 58% of the esophageal cancers and correlated with lymph node metastasis and lymphatic invasion (18). Among our patients, *PDGFRβ* transcription was observed in the vast majority of the tumor samples. However, analysing the location of the expression in a limited number of samples by IHC, we detected it only in the tumor stroma and not in tumor cells themselves. To the best of our knowledge, no data are available correlating *PDGFRβ* or *PDGFRα* expression with the clinical outcome of patients. Most notably, in other tumor entities such as Ewing sarcoma and breast cancer, *PDGFRα* and *PDGFRβ* expression strongly correlated with an invasive behaviour (19,20). *PDGFRα* expression as well as activating mutations have been reported in gastrointestinal stromal tumors (GIST) (21). The high expression rate of *PDGFRα* among our patients implies a similarly relevant role in esophageal cancer. Current publications indicate that *PDGFRα*-mutated GIST displayed an epitheloid or mixed phenotype and were exclusively located in the stomach, whereas *PDGFRα* wild-type tumors occurred in the small bowel (22). Several studies have proven the impact of *EGFR1* expression in tumor proliferation, lymphatic dissemination and poor survival as well as the benefit of EGFR1 inhibition in esophageal adenocarcinoma and ESCC (23-27).

Among our patients, the expression rates for VEGFR1-3, *PDGFRα-β* and *EGFR1* were 97, 94, 77, 91, 86 and 97% for EADC and 100, 100, 53, 100, 87 and 100% for ESCC, respectively. As depicted in Fig.1B, the detection of RTKs was more sensitive by PCR than by IHC, in particular as we applied relatively high cycle numbers in order to identify marginal RTK expression. We chose this procedure as it is a subject of discussion whether tumors are more dependent on RTK-mediated signalling when a specific RTK expression is high or low. It can be hypothesized that each particular receptor is more relevant when expressed at a low level. In contrast, a low receptor number could also indicate non-relevance for the respective tumor cell. Our PCR analyses reported a medium-strong VEGFR1 and VEGFR2 expression in 66 and 60% of ESCC, respectively. These data resemble the IHC expression profile by Kato and colleagues and indicate that IHC may miss the detection of low RTK expression (9). In addition, PCR analyses amplify RTKs expressed in the tumor bed and thus exagerate the expression rate of the tumor as quantified by IHC. However, as RTK inhibitors target RTKs not only in cancer cells, but also in endothelial cells and pericytes, a differentiation of the origin of RTK transcription may be unnecessary as RTK-inhibitors will impact on the tumor bed as a total.

Among our patients, 94% of adenocarcinoma and 100% of squamous cell cancer specimen expressed at least four out of six RTKs. Thus, the vast majority of samples revealed a high frequency of RTK co-expression, indicating their high relevance for tumor progression. In contrast, only 2% of the EADC specimen exhibited no RTK expression at all. So far, analyses of co-expression patterns in human malignancies have only been published for gastric adenocarcinoma (28). Herein, we report the first co-expression profile of drug-targeted RTKs in human esophageal cancer. A correlation with clinicopathological parameters will be performed after the inclusion of more patients in this study.

Our results may encourage the application of tyrosine kinase inhibitors, such as sunitinib or sorafinib, in esophageal cancer as a promising novel approach for innovative treatment strategies. Therefore, we suggest an application of RTK inhibitors in a larger prospective study, analyzing its impact in esophageal cancer patients within a multimodal concept.

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