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/β 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%), PDGFRβ (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%), PDGFRβ (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, PDGFRβ 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.

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 (neoadjuvant 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 search for innovative therapies.

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 (EGFR/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 esophageal 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 with haemalaun solution (Dako).

RNA isolation and reverse transcription-PCR. RNA isolation was performed using an RNAlater kit according to the manufacturer's recommendations (Quagen, Hilden, Germany). The transcription of β-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 β-actin forward: 5'-TGA CGG GTG CAC CAC CCA CAC TGT CAT CTA-3' and reverse 5'-CTA GAA GCA TTT GGC GTG GAC GAC GGA GGG-3' [661 base pairs (bp) fragment], VEGFR1 forward: 5'-TGG GAC AGT AGA AAG GAA CCC CTC GA-3' and reverse 5'-CCC ACG CAG ACA TCA AGA CG-3' and reverse: 5'-TCC AGA ACT CCA CGA TCA CC-3' (380 bp), PDGFRα forward: 5'-CTC CTG AGA GGA TCT TGG 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 GCA GTG ACT GCC TTC-3' (547 bp) and EGFR1 forward: 5'-TCT CAG CAA CAT GTC GAGA-3' and reverse: 5' CGC ACT 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: initial denaturation (4 min at 94°C) followed by the respective number of cycles (β-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; β-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 gradings: 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, PDGFRα) 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, PDGFRβ) 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.
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

Table 1. Antibody characteristics.

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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.
studied, the expression of the ligands VEGFA, VEGFB, with the depth of tumor invasion, tumor stage and lymphatic vessels in esophageal cancer (10,11). As expected, tumor blood vessels 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, PDGFRαβ 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 PDGFRαβ 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 PDGFRβ was significantly higher in tumor tissues than in para-tumoral and normal tissues (16). Matching these observations, imatinib induced apoptosis in PDGFRβ-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 PDGFRα or PDGFRαβ 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, PDGFRαβ 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 exaggerate 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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References