

Coexpression of receptor-tyrosine-kinases in gastric adenocarcinoma-a rationale for a molecular targeting strategy?

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receptor-tyrosine-kinases coexpression in gastric adenocarcinoma and might therefore encourage an application of multiple-target RTK-inhibitors within a combination therapy.

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Abstract

AIM: To define the (co-)expression pattern of target receptor-tyrosine-kinases (RTK) in human gastric adenocarcinoma.

METHODS: The (co-)expression pattern of *VEGFR1-3*, *PDGFR α/β* and *EGFR1* was analyzed by RT-PCR in 51 human gastric adenocarcinomas. In addition, IHC staining was applied for confirmation of expression and analysis of RTK localisation.

RESULTS: The majority of samples revealed a *VEGFR1* (98%), *VEGFR2* (80%), *VEGFR3* (67%), *PDGFR α* (82%) and *PDGFR β* (82%) expression, whereas only 62% exhibited an *EGFR1* expression. 78% of cancers expressed at least four out of six RTKs. While *VEGFR1-3* and *PDGFR α* revealed a predominantly cytoplasmatic staining in tumor cells, accompanied by an additional nuclear staining for *VEGFR3*, *EGFR1* was almost exclusively detected on the membrane of tumor cells. *PDGFR β* was restricted to stromal pericytes, which also depicted a *PDGFR α* expression.

CONCLUSION: Our results reveal a high rate of

INTRODUCTION

Although the incidence of gastric carcinoma has decreased steadily in many industrial nations, it remains among the most frequent malignancies and the common causes of cancer-related deaths worldwide^[1-3]. Radical surgery accompanied with D2-lymph node dissection represents the only curative option and is the standard therapy for patients with limited disease. Despite the improvements of radical primary tumor resection and perioperative therapy, lymphatic and distant recurrence rates remain critical^[4,5].

Accepted risk factors for gastric cancer are chronic atrophic gastritis, chronic *H pylori* infection, hypertrophic gastropathy and Warthin-Lynch syndrome among others^[6]. Different pathogenic alterations instrumentally mediate progression and metastasis of gastric cancer. Molecular determinants occurring during the development of gastric cancer include mutations of certain tumor suppressor genes (*APC*, *DCC*, *Rb*, *p53*), oncogenes (*K-ras*) and repair genes (*MLH-1*)^[7-10].

Receptor tyrosine kinases (RTKs) are transmembrane proteins containing extracellular ligand-binding domains and intracellular catalytic domains^[11]. Receptor binding of the respective ligand results in RTK-autophosphorylation and a consecutive *Mek1/2* and *Erk1/2* activation *via Raf*

or $Ras^{[12,13]}$. Expression of functional receptors and their respective ligands by tumor cells also raises the possibility of autocrine loops and is critical for auto-stimulation and progression^[14,15].

Hitherto, the growth factors *VEGF* and *PDGF* and their receptors have been considered relevant in the process of angiogenesis and dissemination in gastric adenocarcinoma, whereas *EGF/EGFR* was correlated with tumor growth and local invasion^[16-20]. As part of the tyrosine kinase family, *PDGF* receptors are involved in multiple tumor-associated processes, like enhancing tumor angiogenesis by recruitment and regulation of tumor fibroblasts and pericytes^[21]. Data correlating *PDGFR α* or *PDGFR β* expression with clinical outcome in human gastric adenocarcinoma are to the best of our knowledge not available.

As new multi-target tyrosine kinase inhibitor are emerging and enriching the therapy in various malignancies, our aim was to define the expression pattern of target RTKs in human gastric adenocarcinoma and thus elucidate a rationale for a possible new therapeutic strategy^[22,23].

MATERIALS AND METHODS

Tissue source and storage

Tumour samples were obtained from 51 patients undergoing elective surgery for gastric adenocarcinoma at the University of Mainz between 2005 and 2006. Specimens were conventionally stored in formalin for consecutive histopathological analysis. In addition to 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. Those tumour tissue originated from the centre of the tumour. As control tissues, samples of healthy gastric mucosa were collected from the margin of gastrectomy specimens, performed due to non-malignant disease. Informed consent was obtained before the respective tissue was collected.

Immunohistochemistry

Paraffin-embedded tissue samples of normal gastric mucosa and gastric adenocarcinoma were generously provided by S Biesterfeld the Institute of Pathology, University of Mainz and were screened for *VEGFR1-3*, *PDGFR α* / β and *EGFR1* protein expression by immunohistochemistry (Table 1). The tissues were deparaffinized, rehydrated and subsequently incubated with the respective primary antibody (Table 1). The secondary antibody (anti-rabbit-mouse-goat-antibody) was incubated for 15 min at room temperature, followed by an incubation with streptavidin-POD (DAKO, Germany) for 15 min. Antibody binding was visualized using AEC-solution (Dako, Germany). Finally, the tissues were counterstained by hemalaun solution (DAKO, Germany).

RNA isolation and RT-PCR

RNA isolation was performed using the RNeasy Kit according to the manufacturer's recommendations (Qiagen, Hilden, Germany). Transcription of *β -Actin*, *VEGFR1*, *VEGFR2*, *VEGFR3*, *PDGFR α* , *PDGFR β*

Table 1 Antibody characteristics

Target	Antibody	Dilution	Incubation	
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
PDGFR α	PDGFR α (C-20)	1:200	2 h	Santa Cruz Biotechnology, CA, USA
PDGFR β	PDGFR β (28 E1)	1:200	2 h	Cell Signalling Technology, MA, USA
EGFR1	AM207-5ME	1:1	1 h	BioGenex, CA, USA

and *EGFR1* was analyzed by a two-step RT-PCR: reverse transcription was performed with 2 μ g of RNA (20 μ L total volume; Omniscript RT Kit, Qiagen) according to the recommendations of the manufacturer. In total, 0.5 μ L of the cDNA (50 ng) were used as template for the specific PCR reactions. Primers applied were *β -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), *PDGFR α* -forward: 5'-CTC CTG AGA GCA TCT TTG AC-3' and reverse 5'-AAG TGG AAG GAA CCC CTC GA-3', *PDGFR β* -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: initial denaturation (4 min at 94°C) followed by the respective number of cycles (*β -Actin*: 28, *VEGFR1*: 36, *VEGFR2*: 38, *VEGFR3*: 38, *PDGFR α* : 40, *PDGFR β* : 36 and *EGFR1*: 38) of denaturation (1 minute at 94°C), annealing (45 s; *β -Actin*: 52°C, *VEGFR1*: 60°C, *VEGFR2*: 62°C, *VEGFR3*: 62°C, *PDGFR α* : 57°C, *PDGFR β* : 64°C and *EGFR1*: 60°C) and elongation (1 minute 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. Seven μ L of the product were run on a 1.8% agarose gel, stained by ethidium bromide, and analyzed under UV light. The evaluation of expression was performed semiquantitatively according to following grades: negative: 0, weak: 1, medium: 2, strong: 3.

RESULTS

Immunohistochemical staining of receptor tyrosine kinases in gastric mucosa and adenocarcinoma

Negative controls of gastric mucosa and adenocarcinoma

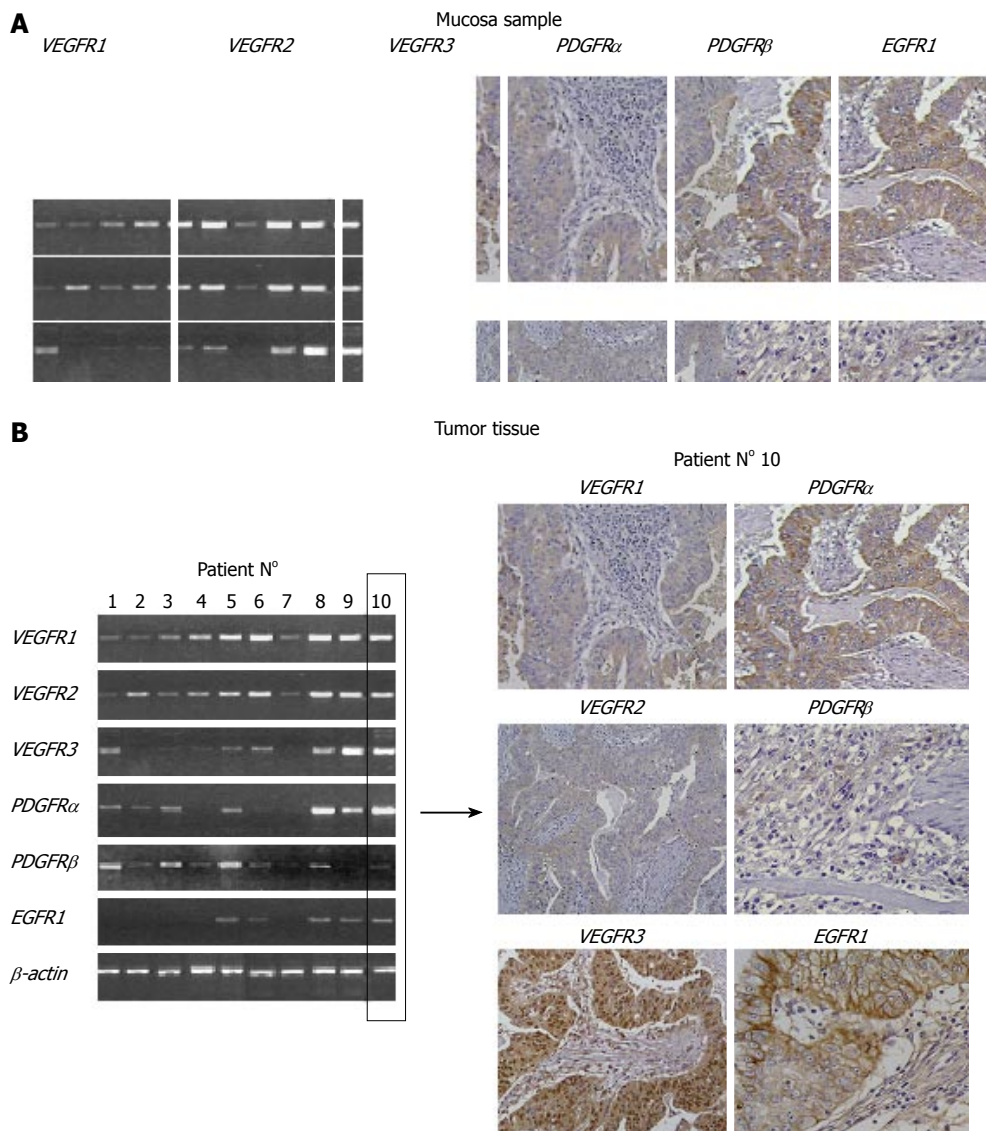


Figure 1 A: The IHC staining of healthy gastric mucosa for VEGFR1-3, PDGFR- α/β and EGFR1. While healthy gastric mucosa revealed a significant EGFR1 and an intermediate VEGFR2 expression in gastric epithelial cells, all other RTKs exhibited only faint to absent expression levels; B: The exemplary transcription profile of 10 gastric cancers and an immunohistochemical analyses of an adenocarcinoma specimen originating from one patient. While VEGFR1, VEGFR2, VEGFR3 and PDGFR α revealed a predominantly cytoplasmic staining accompanied by an additional nuclear staining for VEGFR3, EGFR1 was almost exclusively restricted to the membrane of tumor cells. Stromal cells revealed expression of PDGFR α and PDGFR β .

remained negative for all samples. RTK expression in healthy gastric mucosa varied from strong (EGFR) to intermediate (VEGFR2) and barely detectable/absent (VEGFR1, VEGFR3, PDGFR α ; PDGFR β , Figure 1A). Analyses by PCR revealed an additional VEGFR1 amplicon which could be ascribed to endothelial cells by IHC staining (Figure 1A).

Cancer cells stained for VEGFR1, VEGFR2, VEGFR3, PDGFR α and EGFR1, whereas stromal cells stained for PDGFR α and PDGFR β . VEGFR1, VEGFR2, VEGFR3 and PDGFR α/β revealed a predominantly cytoplasmic as well as a weak membranous localisation, whereas EGFR1 was almost exclusively found on the membrane (Figure 1B). An additional nuclear staining was only seen for VEGFR3.

Receptor tyrosine kinase expression patterns

VEGFR1, VEGFR2, VEGFR3, PDGFR α , PDGFR β and EGFR1 expression in gastric adenocarcinoma samples revealed varying transcription intensities. VEGFR1 expression was observed in 98% of all samples and varied from strong (50%) to intermediate (34%) and weak (16%; Figure 2A). VEGFR2 expression was found in 80% of all gastric

carcinoma specimens and ranged from weak (39%), to intermediate (15%) and strong (46%). The overall expression rate of VEGFR3 was 67% with a weak expression in 21%, an intermediate expression in 35% and a strong expression in 44%. PDGFR α expression was observed in 82% of all samples. A strong PDGFR α -expression was found in 50%, whereas 29% revealed an intermediate and 21% a weak expression. PDGFR β expression was seen in 82% and varied from weak (29%) to intermediate (29%) and strong (42%). The expression rate of EGFR1 was 62% and varied from weak (28%), to intermediate (36%) and strong (36%).

Receptor tyrosine kinase co-expression and correlation with clinicopathological parameters

36% of samples revealed a coexpression of 6 receptors, 28% of 5 receptors, 14% of 5 receptors and only 34% showed co-expression of 3 receptors or less (Figure 2B). Co-expression of VEGFR1, VEGFR2 and VEGFR3 was found in 63% of samples.

DISCUSSION

This is the first study analyzing the (co-)expression profile

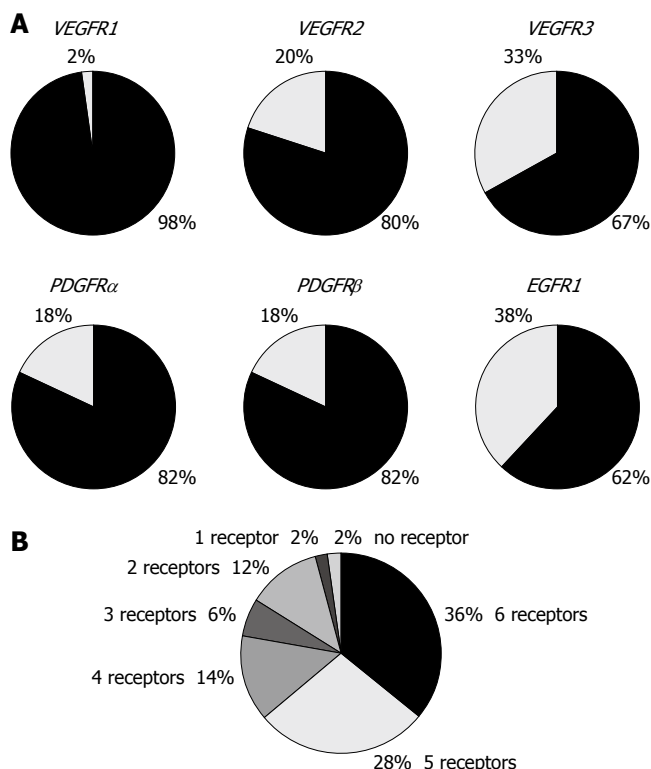


Figure 2 A: The expression profile of RTKs *VEGFR1-3*, *PDGFR α/β* and *EGFR1* in human gastric adenocarcinoma; B: The co-expression rates of those RTKs.

of a series of receptor tyrosine kinases in human gastric adenocarcinoma. We initiated this study while a series of novel multi-target RTK-inhibitors are emerging and enriching classical chemotherapy strategies in order to estimate the benefit of such a therapy in gastric cancer. Our analysis was based on the assumption 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.

VEGFR1-3, *PDGFR α/β* and *EGFR1* undergo phosphorylation following ligand binding resulting in tyrosine kinase activity and concomitant activation of *RAS-Raf-MEK1/2-ERK1/2* pathways^[12,13]. Depending on the location of the RTK on tumor cells, endothelial cells or pericytes, the consequences are tumor cell proliferation, dissemination or angiogenesis.

VEGFR1 and *VEGFR2* are expressed on endothelial cells, whereas *VEGFR3* is largely restricted to lymphatic endothelial cells. While *VEGFR1* expression in gastric adenocarcinoma has been associated with tumor proliferation and dissemination^[24], *VEGFR3* expression has been correlated with lymphatic dissemination in gastric adenocarcinoma^[17]. In a recent study, *VEGFR2* expression did neither correlate with prognosis or dissemination in gastric adenocarcinoma^[25]. Several studies have proven the impact of *EGFR1* expression on poor survival not only in gastric adenocarcinoma^[20,26]. *PDGFR α* expression has been described by Tsuda and colleagues in 8 out of 15 gastric adenocarcinomas analyzed, supporting our results^[27]. No further groups have reported on *PDGFR α* expression in gastric adenocarcinoma, since. Data correlating the

PDGFR α expression with clinical parameters in gastric adenocarcinoma are not available. However, *PDGFR α* expression and activating mutations have been reported in gastrointestinal stromal tumors (GIST)^[28]. Most interestingly, *PDGFR α* mutated GIST displayed an epitheloid or mixed phenotype and were exclusively located in the stomach, whereas *PDGFR α* wildtype tumors also occurred in the small bowel^[29].

PDGFR β expression in gastric adenocarcinoma was only described once in 1992 by Chung and colleagues analyzing three tumor samples^[30]. As to the best of our knowledge, no data are available correlating *PDGFR β* expression with the clinical outcome of patients. However, in other tumor entities such as Ewing sarcoma and breast cancer, *PDGFR α* and *PDGFR β* expression strongly correlated with an invasive behaviour^[31,32].

So far, analyses of coexpression patterns in human malignancies have not been performed previously. Among our patients the majority of gastric adenocarcinoma samples revealed an expression of *VEGFR1-3* and *PDGFR α/β* , whereas two thirds exhibited an *EGFR1* expression.

As expected, *PDGFR α* and *PDGFR β* staining was detected in the tumor stroma and can most likely be allocated to pericytes. While *PDGFR β* was restricted to stromal cells only, *PDGFR α* expression could also be detected in cancer cells. In contrast, *VEGFR1-3* and *EGFR1* expression was restricted to tumor cells. Hence, tumor cells might engage these RTKs in order to emerge to a proliferative and migratory phenotype.

PCR analyses might also amplify RTKs transcribed by vascular and lymphatic endothelial cells or pericytes in the tumor bed. However, as RTK-inhibitors target RTKs not only on cancer cells, but also on endothelial cells and pericytes, differentiation of the origin of RTK-transcription might be unneeded as RTK-inhibitors will impact on the tumor bed as total.

Endocytosis and translocation of RTKs to the perinuclear *rab4*-, *rab5*- and *rab11*-compartments of receptor recycling has previously been reported for diverse membrane receptors helping to explain the cytoplasmatic location as a consequence of receptor-activation^[33,34]. In addition, *VEGFR2* is held in an endosomal storage pool and can be delivered to the plasma membrane upon induction^[35].

Most strikingly, 64% of all cancers analyzed expressed at least five out of six receptor-tyrosine-kinases. Thus, the majority of cases revealed a high frequency of RTK co-expression. In contrast, only 2% of the specimens exhibited no RTK expression at all. Co-expression of *VEGF receptors 1-3* was found in 63% of all samples, indicating a relevant role of the vascular and lymphatic endothelial growth factor system in gastric adenocarcinoma. In summary, we report the first coexpression profile of target receptor-tyrosine-kinases in a solid human malignancy.

These results might encourage the application of multiple-targeted RTK-inhibitors in gastric adenocarcinoma as part of a multidrug therapy. In order to evaluate the impact of multi-targeted RTK-inhibitors we currently ran a phase II study analyzing the benefit of Sunitinib^[36], targeting the above analyzed RTKs in CPT-11 or Cisplatin re-

fractory, disseminated gastric adenocarcinoma. Hereafter, RTK co-expression will be correlated as well with clinical and pathological parameters as with clinical response in gastric adenocarcinoma patients.

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