SPANDIDOS PUBLICATIONS

PDGFRα/ß expression correlates with the metastatic behavior of human colorectal cancer: A possible rationale for a molecular targeting strategy

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Received October 17, 2007; Accepted November 30, 2007

Abstract. As new multi-target tyrosine kinase inhibitors are emerging in the therapy of various malignancies, our aim was to define the co-expression pattern of receptor-tyrosine-kinase platelet-derived growth factor receptors α and ß (*PDGFRα/ß)* in human colorectal cancer. The co-expression pattern of *PDGFRα/ß* was analyzed by RT-PCR in 99 histologically confirmed human colorectal carcinomas and five colorectal cancer cell lines. In addition, immunohistochemical (IHC) staining was applied for confirmation of expression and analysis of receptor tyrosine kinase (RTK) localisation. The colorectal cancer cell lines that were analysed revealed varying expression intensities of *PDGFRα* and *PDGFRß*. The majority of human colorectal cancer specimens revealed a *PDGFRα* (83%) or *PDGFRß* (60%) expression. While *PDGFRα* showed a predominantly cytoplasmic staining in tumor cells as well as in stromal pericytes, *PDGFRß* was restricted to stromal pericytes only. Furthermore, *PDGFRα* expression significantly correlated with lymph node metastasis (P=0.0082) and advanced UICC stages III/IV (P=0.018) in older patients (P=0.043). *PDGFRß* expression only revealed a trend towards lymphatic dissemination (P=0.099). Co-expression of *PDGFRα/ß* occurred in 57% of the colorectal cancer samples, whereas another 29% of the samples depicted mono-expression of *PDGFRα* or *PDGFRß*. Notably, *PDGFRα/ß* expression significantly correlated with lymphatic metastasis (P=0.007) and advanced UICC stages III/IV (P=0.017) in older patients (P=0.03). In summary, our results revealed that PDGFRα/ß expression significantly correlates with lymphatic dissemination and therefore encourages application of PDGFRα/ß RTK-inhibitors within a combination therapy.

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Abbreviations: bp, base pair; CRC, colorectal carcinoma; IHC, immunohistochemistry; PCR, polymerase chain reaction; PDGF, platelet-derived growth factor; PDGFR, platelet-derived growth factor receptor; RTK, receptor tyrosine kinase; OTK, oncogenic tyrosine kinase

Key words: lymph node metastases, distant metastases, progression, colorectal cancer, platelet-derived growth factor receptor

Introduction

Colorectal cancer (CRC) is among the most frequent cancers in western countries and is delineated by local recurrence, lymphatic and hematogenous dissemination (1-3). Less frequently, the occurrence of CRC is due to hereditary syndromes like familial adenomatous polyposis (FAP) and hereditary non-polyposis colon cancer (HNPCC). However, the majority of CRC patients (85%) suffer from sporadic carcinogenesis (4). Molecular determinants occurring during the development of sporadic CRC include mutations in certain tumor-suppressor genes (*APC*, *DCC*, *Smad-2*, *Smad-4* and *p53*) and oncogenes (K-*ras*) that have been summarized in the adenoma-carcinoma sequence initially proposed by Fearon and Vogelstein (5-7). However, as <10% of colorectal cancers harbor concomitant mutations of *APC*, *K-ras* and *p53* it seems very likely that additional pathogenic alterations are instrumental in mediating progression and metastasis of colorectal cancer (8).

Tumor growth and metastatic dissemination are considered to be a consequence of a complex, dysregulated molecular machinery. Receptor tyrosine kinases (RTK) containing extracellular ligand-binding domains and intracellular catalytic domains, are known to contribute to these phenomena via

their signaling pathways including the *Ras-Raf-MAPK* cascade and the PI-3K pathway (9-13). Therefore, RTKs are also called oncogenic tyrosine kinases (OTKs) (14). Constitutive activation of OTKs either by gene alteration, overexpression or defective downregulation protects tumor cells from apoptosis, while enabling their invasion and metastasis (10,15). Another mechanism endowing tumors with proliferative and metastatic potential is the existence of autocrine loops leading to auto-stimulation of RTK/RTL-ligand expressing cells, as also shown for *PDGFR* (16-18). In this respect, PDGFs and their corresponding receptors have been considered relevant in the process of (lymph-) angiogenesis and dissemination in colon carcinoma (19). In particular, *PDGFs* and their respective receptors enhance neo-(lymph) angiogenesis in malignomas by recruitment and induction of proliferation of tumor fibroblasts and pericytes (20).

However, data correlating *PDGFRα* or *PDGFRβ* expression with the clinical outcome in human colorectal carcinoma are to the best of our knowledge not available. As new multi-targeted tyrosine kinase inhibitors are emerging in the therapy of various malignancies, our aim was to define the expression pattern and clinical relevance of *PDGFRα/β* in human colorectal carcinoma and thus elucidate a rationale for a possible new therapeutic strategy (21,22).

Materials and methods

Cell culture. The human colorectal cancer cell lines SW480, SW620, Caco-2, HDC8 and HT29 were cultured in DMEM (Invitrogen, Germany) supplemented with 10% FCS, 100 units/ml penicillin, 100 μg/ml streptomycin (Cambrex, Germany) and 1 mM L-glutamine (Invitrogen).

Tissue samples. Colorectal cancer tissue samples were obtained from 99 consecutive patients undergoing elective surgery for colorectal cancer. The tumor tissue originated from the center of the tumor. Informed consent was obtained before the respective tissue was collected. All tissues were stored in cryovials, shock-frozen in liquid nitrogen immediately after extirpation and stored at -80˚C until further processing. The morphological classification of the carcinomas was conducted according to World Health Organization (WHO) specifications.

RNA isolation and semiquantitative RT-PCR. RNA isolation was performed using the 'Qiagen RNeasy Kit' according to the manufacturer's recommendations (Qiagen, Hilden, Germany). Gene transcription of *β-Actin*, *PDGFRα* and *PDGFRβ* was analyzed by a two-step RT-PCR: Reverse transcription was performed with 2 μg of RNA (20 μl total volume; Ominscript RT kit, Qiagen, Germany) according to the recommendations of the manufacturer. The cDNA (0.5 μl) (50 ng) was used as a template for the specific PCR-reactions. Primers applied were *β-Actin*-forward: 5'-TGA CGG GGT CAC CCA CAC TGT GCC CAT CTA-3', *β-Actin*-reverse: 5'-CTA GAA GCA TTT GCG GTG GAC GAC GGA GGG-3' [661 base pairs (bp) fragment], *PDGFRα*-forward: 5'-CTC CTG AGA GCA TCT TTG AC-3' and *PDGFRα*-reverse 5'-AAG TGG AAG GAA CCC CTC GA-3' (712 bp), *PDGFRβ*-forward: 5'-TCC TCA ATG TCT CCA GCA CCT TC-3' and *PDGFRβ*-reverse 5'-ACC ACA GTC TGC ACT GCG TTC-3' (547 bp). For amplification, a 'DNA Engine PTC200' (MJ Research, Watertown, USA) thermocycler was used. Cycling conditions of the respective PCR were as follows: initial denaturation (4 min at 94˚C), followed by the respective number of cycles (*β-Actin*: 28; *PDGFRα*: 38 and *PDGFRβ*: 38) of denaturation (45 sec at 94˚C), annealing (45 sec; *β-Actin*: 52˚C; *PDGFRα*: 57˚C, *PDGFRβ*: 64˚C) and elongation (1 min and 30 sec 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 products (8 μl) were run on a 1.8 % agarose gel, stained by ethidium bromide and analysed under a UV light.

Western blot analysis. SW480, SW620, Caco-2, HDC8 and HT29 cells were washed with PBS and lysed in 0.5% NP-40 solution. For *α-Tubulin* control (20 μg) and for *PDGFRα/β* analyses (80 μg) protein was loaded on a 10% SDS-PAGE gel. The gel was transferred onto a PVDF membrane following seperation. The respective proteins were detected with anti-

Table I. Patient and tumor characteristics.

	Patient characteristics
Total number	99
Median age (years)	65.4
Gender	
Female	42 (42%)
Male	57 (58%)
Location	
Colon	64 (65%)
Rectum	35 (35%)
UICC	
1	16 (16%)
2	40 (41%)
3	21 (21%)
4	22 (22%)
T-status	
1	5 (5%)
2	16 (16%)
3	63 (64%)
4	15 (15%)
N-status	
0	59 (60%)
1	21 (21%)
2	19 (19%)
M-status	
0	77 (78%)
1	22 (22%)
R-status	
0	76 (94%)
1/2	23 (4%)

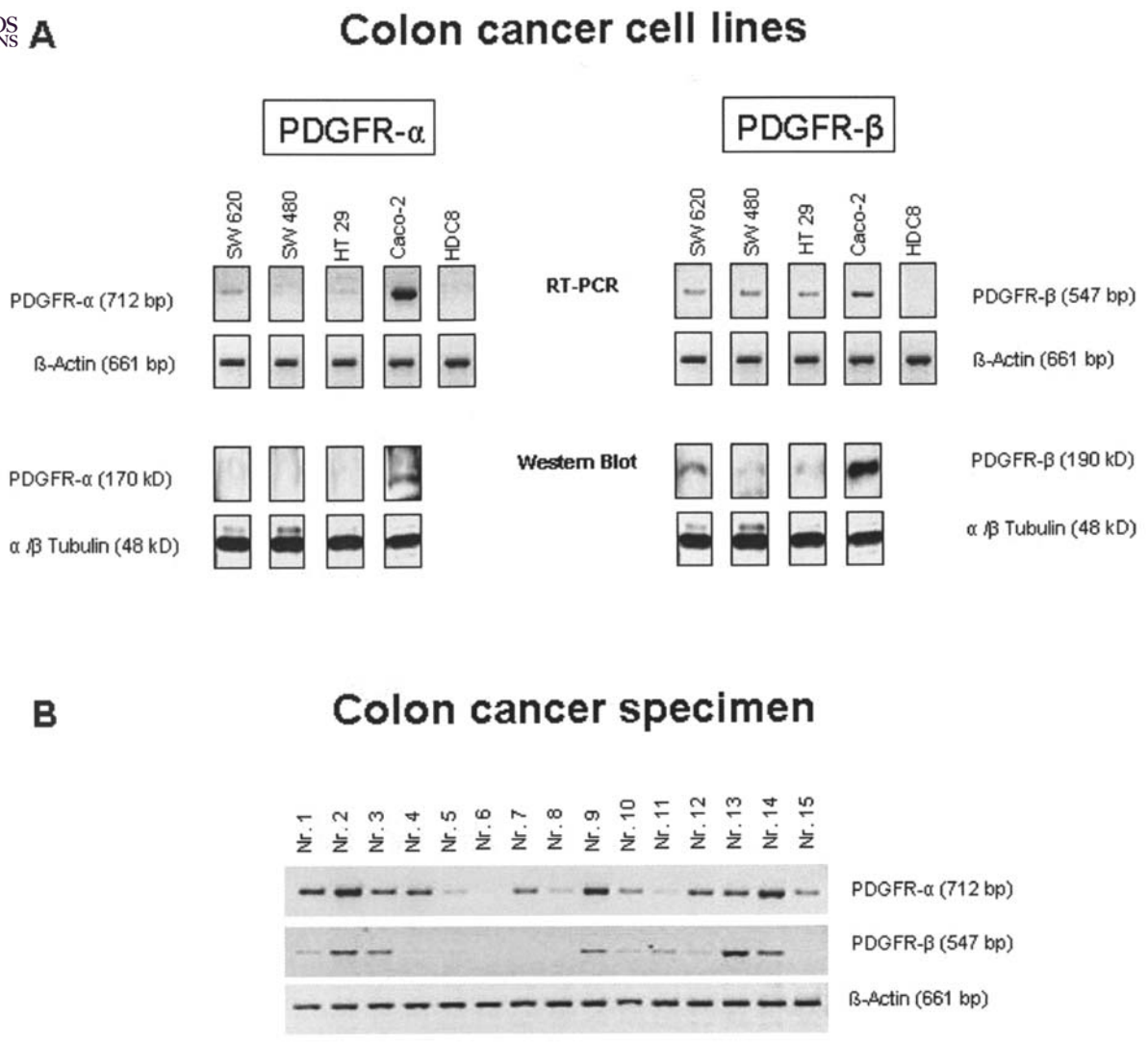

Figure 1. Expression and transcription profile of PDGFRα and PDGFRß in diverse human colorectal cancer cell lines (A). Co-expression profile of PDGFRα and PDGFRß in 15 colorectal cancer samples (B).

PDGFRα (1:300, rabbit anti-human polyclonal IgG 200 μg/ml, C-20, Santa Cruz Biotechnology, USA; 1:10000 goat anti-rabbit IgG 2nd antibody HRP 200 μg/0.5 ml by Santa Cruz Biotechnology), anti-*PDGFRß* (1:1000, rabbit anti-human monoclonal IgG, 28E1, Cell Signaling Technology, USA; 1:10000 goat anti-rabbit IgG 2nd antibody HRP, 200 μg/0.5 ml by Santa Cruz Biotechnology) and *α-Tubulin* (1:2000, mouse anti-human monoclonal IgG, B-5-1-2, Sigma-Aldrich, Germany; 1:10000, goat anti-mouse IgG 2nd antibody, HRP 200 μg/0.5 ml, Santa Cruz Biotechnology) and were visualized by an ECL Western blotting analysis system (Amersham Biosciences, USA).

Immunohistochemistry. Ten paraffin-embedded tissue samples of normal colon mucosa and colon carcinoma were generously provided by S. Biesterfeld (Institute of Pathology, University of Mainz) and were screened for *PDGFRα* and *PDGFRß* protein expression by immunohistochemistry. The tissues were deparaffinized, rehydrated and subsequently incubated with the respective primary antibody [anti-*PDGFRα* (C-20); 1:200, 2 h, Santa Cruz Biotechnology, CA, USA and anti-*PDGFRß* (28E1), 1:200, 2 h, Cell Signalling Technology, MA, USA]. 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). Then, the tissues were counterstained by haemalaun solution (Dako).

Statistics. The association of staining intensity with clinicopathological patterns was assessed with the χ^2 test and with the unpaired Student's t-test, when appropriate. $P<0.05$ was considered significant and $P<0.001$ highly significant in all statistical analyses.

Results

Expression in colorectal carcinoma cell lines. *PDGFRα* expression and transcription of human colorectal cancer cell lines varied from strong (Caco-2) to weak (SW620, HT29) and absent (SW480, HDC8) as depicted in Fig. 1A. Similarly, *PDGFRß* expression and transcription varied from intermediate (Caco-2) to weak (SW620, SW480, HT29) and absent (HDC8). *PDGFRα/ß* transcription and expression analyses resulted in a single specific band, respectively.

Table II. Patient and tumor characteristics dependent on *PDGFRα* expression.

	PDGFRα expression		
	Absent	Present	Statistics
Total number	17 (17%)	82 (83%)	
Median age (years)	60.9	66.4	P=0.043
Gender			
Female	7 (41%)	35 (43%)	P=0.908
Male	10 (59%)	47 (57%)	
Location			
Colon	10 (59%)	54 (66%)	P=0.58
Rectum	7 (41%)	28 (34%)	
UICC			
I/II	14 (82%)	42 (51%)	P=0.018
III/IV	3 (18%)	40 (49%)	
T-status			
1+2	5 (29%)	16 (19%)	P=0.349
3+4	12 (71%)	66 (81%)	
N-status			
0	15 (88%)	44 (54%)	P=0.0082
+	2 (12%)	38 (46%)	
M-status			
0	15 (88%)	62 (76%)	P=0.254
+	2 (12%)	20 (24%)	
R-status			
0	14 (82%)	62 (76%)	P=0.54
+	3 (18%)	20 (24%)	

Tumor characteristics and patient profiles. The selected group of patients represents typical characteristics of colorectal cancer in industrialized countries, except for a lower percentage (35%; normal expectation 50%) of rectal cancers (Table I).

PDGFRα expression in colorectal cancer. *PDFGRα* expression was observed in 83% of colorectal cancer samples and significantly correlated with lymphatic dissemination (N-status; P=0.0082) and progressed UICC stages (UICC III/IV; P=0.018) as compared to UICC stages I/II, describing limited disease (Fig. 1B, Table II). Patients with *PDFGRα*-positive tumors were of more advanced age (66.4 years) than those with *PDFGRα*-negative cancers (60.9 years; P=0.043). *PDFGRα* expression did not correlate with the T- or M-status, nor with gender, location or the resection-status (R-status).

PDGFRβ expression in colorectal cancer. *PDGFRβ* expression was observed in 60% of colorectal cancer samples and revealed a trend towards lymphatic dissemination (N-status; P=0.099) and progressed UICC stages (UICC III/IV; P=0.163) as compared to UICC stages I/II, describing limited disease (Fig. 1B, Table III). Similarly, PDGFRß expression did not correlate with the T- or M-status, nor with age, sex, location or the resection-status (R-status).

PDGFRα/β co-expression in colorectal cancer. Co-expression of *PDGFRα/β* occurred in 57% of colorectal cancer samples whereas another 29% of samples depicted mono-expression of *PDGFRα* or *PDGFRβ* (Table IV). Notably, any *PDGFRα/β* expression significantly correlated with lymphatic metastasis (P=0.007) and progressed UICC stages III/IV (P=0.017) as compared to UICC stages I/II describing limited disease. Patients with *PDGFRα/β*-positive tumors were older (PDGFR mono-expression: 67.6 years; co-expression: 65.7 years) than those with *PDGFRα/β*-negative cancers (59.7 years; P=0.03). Evidently, *PDGFRα/β* expression did not have an impact on T-, M- or R-status (resection-status) nor on gender or location of the primary tumor.

Immunohistochemical staining of PDGFRα and PDGFRβ. Negative controls of colon mucosa and colorectal carcinoma samples remained negative for all samples (Fig. 2A and B). In healthy colon mucosa, cytoplasmic *PDFGRα* expression was readily detectable, whereas *PDGFRβ* expression was absent. Among all *PDFGRα*-positive samples, *PDFGRα* revealed a cytoplasmic staining of tumor cells in 100% of the specimens

Patient and tumor characteristics dependent on *PDGFRβ* expression.

	PDGFRβ expression		
	Absent	Present	Statistics
Total number	40 (40%)	59 (60%)	
Median age (years)	65.0	65.7	P=0.93
Gender			
Female	18 (45%)	24 (41%)	P=0.66
Male	22 (55%)	35 (59%)	
Location			
Colon	24 (60%)	40 (68%)	P=0.42
Rectum	16 (40%)	19 (32%)	
UICC			
I/II	26 (65%)	30 (51%)	P=0.163
III/IV	14 (35%)	29 (49%)	
T-status			
1+2	11 (28%)	10 (17%)	P=0.207
3+4	29 (72%)	49 (83%)	
N-status			
0	28 (70%)	31 (53%)	P=0.099
+	12 (30%)	28 (47%)	
M-status			
0	32 (80%)	45 (76%)	P=0.66
+	8 (20%)	14 (24%)	
R-status			
0	30 (75%)	46 (78%)	P=0.73
+	10 (25%)	13 (22%)	

and of stromal cells in 70% of the specimens. *PDGFRβ* remained restricted to stromal pericytes only. Staining intensities varied from absent to weak and strong for cytoplasmic *PDFGRα* expression. Stromal *PDFGRα* and *PDGFRβ* expression varied from absent to a focal and diffuse staining pattern. A nuclear staining of *PDFGRα/β* was not observed.

Discussion

This is the first study analyzing the expression profiles of *PDGFRα* and *PDGFRβ* in a large series of human colorectal cancer tissues and cell lines and correlating expression profiles with clinicopathological parameters. The first description of *PDGFR* expression by colorectal carcinoma cell lines dates back to 1989 (23).

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 colorectal cancer. *PDGFRα/β* are frequently targeted by available small molecules, have been reported to influence lymphatic angiogenesis in other cancers and were therefore chosen for this analysis. We aimed at determining whether *PDGFRα/β* expression similarly influences the metastatic behavior of colorectal cancer in patients, as recently reported for other tumor entities.

Expression of *PDGFRα/β* has been described in other malignancies such as breast cancer, gastric adenocarcinoma, pancreatic cancer, prostate cancer and ovarian cancer (24-28). In our study, the analyzed human colorectal cancer cell lines revealed different intensities of *PDGFRα* and *PDGFRβ* expression. While *PDGFRα* expression varied from strong to absent, *PDGFRβ* expression was in general weaker and might result from artificial *in vitro* culturing conditions, as *PDGFRβ* expression could not be observed in human colorectal carcinoma cells. However, *PDGFRα/β* transcription levels correlated with the respective expression levels in human colorectal cancer cell lines indicating a high specificity of the antibodies applied.

Normal colon mucosa samples expressed *PDGFRα* though not *PDGFRβ*, as previously reported by Craven and colleagues (29). Immunohistochemical staining of human colorectal cancer specimens displayed a cytoplasmic *PDGFRα* expression as well of tumor cells. The intracellular cytoplasmic enrichment of *PDGFRα* observed in cancer cells might result from an unsuccessful degradation after internalization. This failure could be based on alterations that uncouple *PDGFRα* from c-Cbl-mediated ubiquitination, a

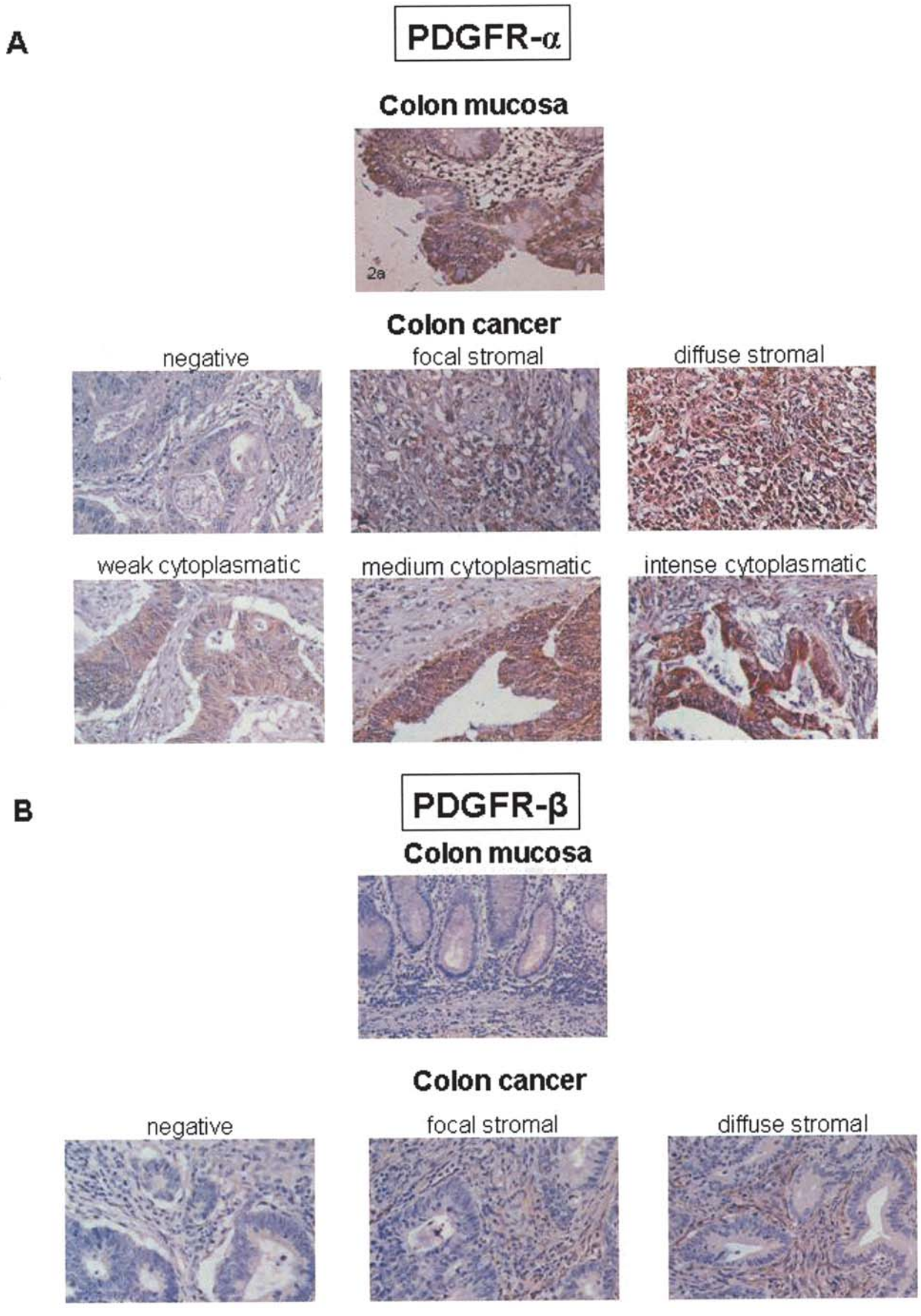

Figure 2. Intensity of PDGFRα and PDGFRß expression in colon mucosa and different colorectal carcinomas. In healthy colon mucosa cytoplasmic PDFGRα expression was readily detectable, whereas PDGFRß expression was absent. Among the PDFGRα-positive samples, PDFGRα revealed a cytoplasmic staining of tumor cells in 100% of the specimens and of stromal cells in 70% of the specimens. PDGFRß remained restricted to stromal pericytes only. Staining intensities varied from absent to weak and strong for cytoplasmic PDFGRα expression. Stromal PDFGRα and PDGFRß expression varied from absent to a focal and diffuse staining pattern. A nuclear staining of PDFGRα/ß was not observed.

	Expression of PDGFRα/ß			
	None	α or ß	α and ß	Statistics
Total number	14 (14%)	29 (29%)	56 (57%)	
Average age (years)	59.7	67.6	65.7	P=0.03
Gender				
Female	6 (57%)	13 (55%)	23 (59%)	P=0.97
Male	8 (43%)	16 (45%)	33 (41%)	
Location				
Colon	8 (57%)	18 (62%)	38 (68%)	P=0.52
Rectum	6 (43%)	11 (38%)	18 (32%)	
UICC-stage				
I/II	12 (86%)	16 (55%)	28 (50%)	P=0.017
III/IV	2 (14%)	13 (45%)	28 (50%)	
T-status				
1+2	5 (36%)	6 (21%)	10 (18%)	P=0.15
3+4	9 (64%)	23 (79%)	46 (82%)	
N-status				
0	13 (93%)	17 (59%)	29 (53%)	P=0.007
+	1 (7%)	12 (41%)	27 (47%)	
M-status				
0	12 (86%)	23 (79%)	42 (75%)	P=0.44
+	2 (14%)	6 (21%)	14 (25%)	
R-status				
0	11 (79%)	22 (76%)	43 (77%)	P=0.86
+	3 (21%)	7 (24%)	13 (23%)	

prerequisite for receptor degradation in the endolysosomal compartment (15). It is known that hampered RTK down-regulation is tightly associated with the pathogenesis of cancer.

In contrast, *PDGFRβ* was restricted to tumor-associated stromal cells and pericytes of tumor vasculature as previously reported by Kitadai *et al*, who also described an increase of *PDGFRβ*-positive stromal cells in highly metastatic tumors as compared to low metastatic tumors (30,31).

The majority of our human colorectal cancer specimens revealed a *PDGFRα* (83%) or *PDGFRβ* (60%) expression as well as an expression of both receptors (86%). These results are supported by a small previous analysis (n=18) reporting a *PDGFRα/β* expression in 67% of colorectal cancer samples (32). However, due to the large number of cases investigated, our results might reflect the real expression rates.

Notably, *PDGFRα* mono-expression as well as *PDGFRα/β* expression were significantly associated with progressed UICC stages III/IV and lymph node metastases in older colorectal cancer patients with sporadic cancers. These findings can be supported by recent studies conducted in other tumor entities, where expression of both, *PDGFRα* and *PDGFRβ*, was found to be correlated with an invasive tumor phenotype, advanced clinical stages and consequently an unfavorable prognosis (18,24,28). However, we found no influence of gender, location, T- or M-status on the *PDGFR* expression pattern. Most notably, the impact of *PDGFRα* expression in colorectal cancer samples was much more relevant for lymphatic dissemination than that of *PDGFRβ*. Data describing the relevance of *PDGFRα* for lymphatic dissemination in colorectal cancer are to the best of our knowledge not available and need to be analyzed urgently. *PDGFRβ* expression, however, has previously been located in tumor stromal cells and pericytes and was correlated with tumor dissemination (30,31).

Previous publications implied important interactions between the tumor and its environment resulting from tumor-prone *PDGF* eliciting responses in *PDFGR*-expressing tumor stromal cells. In particular, *PDGF* acted as a mitogen on stromal fibroblasts and pericytes causing tumor angiogenesis (33,34). In colon carcinoma cells for instance, *TGF-β*-induced *PDGF* was ascribed to act exclusively via paracrine effects on tumor vascularization (19).

The fact that *PDGFRα* is not only expressed by tumor cells but also by the surrounding stromal cells or pericytes in general, makes this receptor attractive for targeted therapies by RTK-inhibitors.

In consequence, RTK-inhibitors negatively affect tumor growth and dissemination, as confirmed by murine *in vivo* studies demonstrating that the inhibition of the *PDGFR* signalling pathways results in a significant reduction of experimental metastasis (18,26). These results encouraged the application of multiple-target RTK-inhibitors in human malignancies. Matching these results, Sunitinib, a multiple-target RTK-inhibitor, demonstrated clinical activity in neuro-endocrine, colon and breast cancers in phase II studies (35).

In conclusion, expression of *PDGFRα/β* in colorectal cancer is significantly associated with lymphatic dissemination and progressed UICC stages III/IV reflecting progressed disease in older patients. Thus, *PDGFRα/β* apparently plays an important role during colorectal cancer dissemination and might be a valuable therapeutic target. Hence, further efforts will be necessary in order to evaluate the inhibition of metastatic growth by *PDGFRα/β* antagonists.

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