Quantification of MET and hepatocyte growth factor/scatter factor expression in colorectal adenomas, carcinomas and non-neoplastic epithelia by quantitative laser scanning microscopy

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Abstract. Hepatocyte growth factor (HGF)-MET signalling in cancer biology has been well characterized in multiple organ systems. Numerous investigations have described an up-regulation of c-met mRNA in human colorectal adenomas and carcinomas. However, a quantitative immunohistochemical analysis of MET and HGF protein levels in tumor tissues has not been reported previously. Formalin-fixed and paraffin-embedded tissues from 41 colorectal adenomas and 49 colorectal carcinomas were characterized by immunofluorescent staining using HGF- and MET-specific antibodies. The immunoreactivity was evaluated by confocal laser scanning microscopy, computer-based image analysis and appropriate statistical tests. Normal colorectal mucosa, adenomas and carcinomas exhibited comparable levels of MET and HGF proteins. MET expression in carcinomas, although statistically not significant, demonstrated a tendency to correlate with the grade of differentiation. Correlations of MET and HGF with other clinico-pathological variables including the extent of the mucinous component and the pTNM stage were not observed. The ratio of HGF in carcinoma vs. non-neoplastic tissue was significantly different between high and low carcinoma stage. Alterations of absolute levels of MET and HGF protein during the colorectal adenoma-carcinoma sequence were not significant. The presumed role of MET-HGF interactions in large bowel carcinogenesis may therefore be a result of or depend upon other regulatory factors involved in MET-mediated signalling pathways.

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Introduction

Colon carcinoma is one of the most common malignant tumors in the industrialized countries (1). The majority of these carcinomas arise in pre-existing adenomas via the so-called adenoma-carcinoma sequence (2) but multiple pathways of development have been proposed. The sequence of genetic alterations occurring during the process of neoplastic and finally malignant transformation has been well characterized (3). Various molecules are involved in this process, initiated by mutation of the APC tumor suppressor gene (4). One of the best characterized signalling pathways involves Wnt/ß-catenin/APC. Interestingly, there is a report on the relationship between MET and Wnt (5) that describes MET expression as an early event in the colorectal adenoma to carcinoma sequence that is controlled by the Wnt pathway. Few reports measure the expression of MET and hepatocyte growth factor/scatter factor (HGF/SF) although Hiscox *et al* have reported on a small series of cases (6).

HGF has been characterized as a growth factor for hepatocytes (7-9) and inducer of cellular motility (10,11). HGF/SF is the ligand for MET (12). MET was originally described as a transforming gene from a cell line treated with a chemical carcinogen (13), related to tyrosine kinase oncogenes (14). HGF/SF levels vary in many pathologic and physiologic processes (15) and are especially important in injury, differentiation and growth. Otte *et al* described the functional expression of HGF and MET in colorectal cancers and suggested a paracrine mechanism of growth enhancement (16). The multiple features and properties of MET and HGF have been described in great detail in the review by Birchmeier *et al* (17).

The MET kinase is composed of a heterodimeric structure with an extracellular 50-kDa α chain and a transmembranous 145-kDa ß chain (18). HGF-MET signalling induces various processes in tumor biology (19), including proliferation (20), cell motility and invasiveness (17,20-22), lumen formation and branching morphogenesis (23-26) as well as angiogenesis and stimulation of endothelial cell proliferation (27). Activating mutations of the c-*met* gene and increased HGF stimulation of the tumor cells may be involved in these effects. The

existence of mis-sense mutations of the met proto-oncogene in exons 16-21 coding for the tyrosine kinase domain was demonstrated in human papillary renal, ovarian and hepatocellular carcinomas (28-33). Furthermore, a mutation in exon 14 coding for the juxtamembranous domain was detected in gastric adenocarcinoma (34). Parr *et al* (35) described an antagonist for HGF/SF, NK4 and showed how it reduced invasion in an *in vitro* assay. Herrera (36) similarly showed how HGF affected scattering *in vitro* using HT 29 colon carcinoma cells. Autocrine HGF stimulation was described in sarcomas and astrocytomas (37,38), paracrine HGF secretion by stromal cells was observed in other cancers (39-41). In colorectal carcinomas, an overexpression of the c-*met* oncogene was reported (42,43) and other authors obtained similar results (44-47). The present study was performed in order to quantify the expression of MET as well as HGF/SF protein expression during the colorectal adenoma-carcinoma sequence. HGF and MET protein were localized by immunofluorescence and their reactivity was detected by confocal laser scanning microscopy. The data were evaluated by quantification of the fluorescence intensity in the regions of interest (non-neoplastic vs. adenoma vs. carcinoma tissue) via computer-based image analysis.

Materials and methods

Tissues. Formalin-fixed, paraffin-embedded tissues from 41 colorectal adenomas and from 49 colorectal carcinomas from the files of the Institute of Pathology of the University of Cologne, Germany, were investigated. According to the WHO classification, 27 adenomas exhibited low-grade and 14 adenomas exhibited high-grade intraepithelial neoplasia (48). The group of colorectal carcinomas consisted of 37 intestinal type adenocarcinomas, 11 mucinous adenocarcinomas (characterized by extracellular mucin in at least 50% of the tumor area) and one solid carcinoma. Staging and grading were performed according to the TNM classification (49). Adjacent normal or reactively altered colorectal tissue was available in 64 cases (29 adenomas, 35 carcinomas).

Monoclonal antibodies and immunofluorescent double staining procedure. A rabbit polyclonal antibody (pab) C-28 directed against human MET (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and a murine monoclonal antibody (mab) detecting human HGF prepared in the Laboratory of Monoclonal Antibody Production, VARI, Grand Rapids, MI, USA, (50) were applied in order to localize the respective molecules. Four-μm thick sections from the tissue blocks were deparaffinized according to routine histological techniques. Non-specific protein binding was blocked by 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) for 30 min at room temperature (RT). Incubation with pab C-28 (4 μg/ml blocking buffer) and anti-hHGF mab (20 μg/ml blocking buffer) followed overnight at 4˚C. Non-specific rabbit and mouse IgG replacing the primary antibodies were used as negative controls. After three-fold washing with PBS/0.5% Tween-20, rhodamine-conjugated donkey anti-rabbit immunoglobulin and FITC-conjugated donkey anti-mouse immunoglobulin (Jackson Laboratory, Bar Harbour, ME, USA) diluted in blocking buffer, were incubated at RT for 30 min. Finally, the slides were washed three times with PBS/0.5% Tween-20 and mounted in Gel/Mount permanent aqueous mounting medium (Biomeda, Foster City, CA, USA).

Quantitative fluorescent analyses. Fluorescent analyses were performed according to previously published methods (51-53). Fluorescent-stained sections were imaged using 488- and 543-μm wavelength excitation for FITC and rhodamine, respectively, using a Zeiss LSM 410 confocal laser scanning microscope (Zeiss, Oberkochen, Germany) configured with a 25-mW argon internal HeNe laser. Nomarski images were produced applying a 635 red laser. All analyses were performed with the same laser intensity, brightness, power and scan rate. Images were captured as 512x512 pixel three channel (RGB) TIFF images. The mean intensity of the red and green channels was calculated, excluding pixels with intensities below the background threshold, as determined computationally by the method of Ridler and Calvard (54). The images were analyzed using an image analysis library we have developed for the R statistical analysis framework (55). Using the Nomarski and H&E images, regions of interest (ROIs) differentiating normal and tumor tissue within each image (where applicable) were manually drawn. Using these ROIs, the ratio between normal and tumor fluorescent intensity for both MET and HGF/SF staining was calculated for each patient. Two images were captured for both tumor and normal tissue (where available) for each lesion, and the mean intensities of the ROIs within these two images were averaged to achieve a final mean fluorescent intensity for each marker (MET and HGF/SF) and tissue type (normal and tumor). This ratio was then log transformed (base 2) to normalize the distribution of ratio values.

Statistical analyses. Mean expression levels of MET and HGF/SF as estimated by fluorescent intensity were compared between the tissue types (normal vs. tumor and adenoma vs. carcinoma) using two tailed t-tests. Likewise, the log-transformed ratio of normal to tumor tissue expression of MET and HGF/SF was compared for adenomas vs. carcinomas. Expression level of these proteins, as well as log-transformed ratio of normal to tumor expression, was also compared stratifying for various clinical parameters including grade (high vs. low) and TNM stage, using t-tests (serially, where necessary). All analyses were performed using the R statistical analysis framework.

Results

Neoplastic and non-neoplastic tissue areas in the specimens were identified by histopathological evaluation of the H&E-stained images alone or Nomarski and H&E combinations. An expression of MET protein was observed in the cytoplasm of tumor cells in most adenomas as well as carcinomas. Similarly, in normal or reactively altered epithelial and interstitial cells a fluorescent staining was present (Fig. 1A-D). On the other hand, HGF/SF staining was mostly confined to cells in the surrounding interstitial tissue, whereas only scattered tumor cells showed immunoreactivity (Fig. 1E and F).

In statistical analyses, no significant differences with regard to MET or HGF/SF expression in neoplastic and non-neoplastic

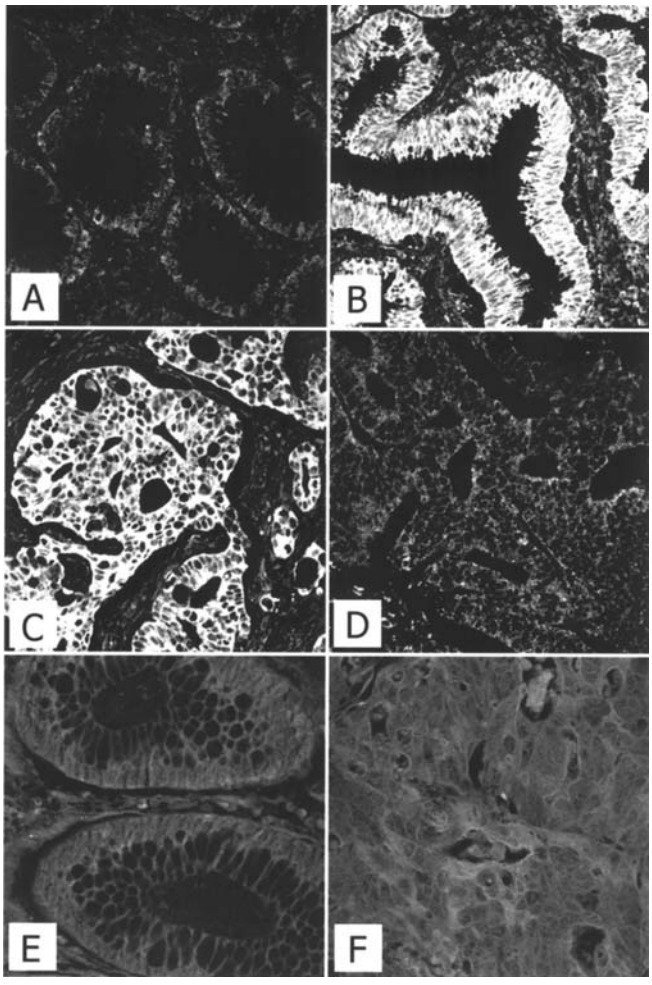

Figure 1. Normal colorectal mucosa exhibiting a faint MET expression (A) and an adenoma with high-grade intraepithelial neoplasia and strong MET immunoreactivity (B). Carcinomas showed a strong (C) or faint (D) MET positivity. HGF was mainly expressed in stromal cells in normal (E) as well as carcinomatous (F) tissue.

epithelia were detected (Table I). Additionally, the expression levels of both molecules did not distinguish between adenomas and carcinomas. Expression of HGF/SF in adenomatous tissue showed a statistically insignificant relationship (p=0.11) with low- vs. high-grade intraepithelial neoplasia, whereas all other parameters did not correlate at all.

Furthermore, fluorescence intensity detection values were correlated with histopathological parameters (Table II). The ratio of HGF expression in tumor vs. non-neoplastic tissues was predictive of high or low pTNM stage (p=0.016). MET expression in carcinomas showed a tendency to correlate with the grade of tumor differentiation which, however, was of borderline significance (p=0.08). The ratio of tumor to normal tissue expression showed a similar result (p=0.16). No other parameters, including HGF/SF expression were significant, or exhibited any significant correlation with carcinoma stages or histopathological subtypes according to the WHO classification.

Discussion

MET overexpression has been described in a variety of human cancers and cell lines derived from brain (56), breast (52,57), ovary (51), nasopharynx (53) and colorectal tissue (43). In the colorectum, multiple investigations have been completed applying different methodical approaches. In most of these studies, mRNA was isolated and measured. In an early investigation, Liu *et al* reported a consistent (mean six-fold) c-*met* mRNA overexpression in carcinomas compared to normal mucosa. Seventy percent of paired normal-tumor specimens showed a tumor to normal c-*met* mRNA ratio of >4 in their study and the expression of c-*met* mRNA was also enhanced in adenomas, suggesting that overexpression of this proto-oncogene may have mechanistic significance in the early stages of human colorectal carcinogenesis (42). A significantly higher c-*met* mRNA concentration in carcinomas compared to normal tissues was confirmed in an investigation involving 22 cases (58). Another group observed an increase of c-met gene expression from 5- to 50-fold in approximately 50% of tumors (n=123), at any stage of progression, and in 70% of liver metastases (n=25). Overexpression was associated with amplification of the c-*met* gene in only 10% of carcinomas, but in 8 of 9 metastases examined suggesting a selective growth advantage mediated by c-*met* (43). Contradictory results were reported by Otte *et al*, who observed a reduced c-*met* and HGF mRNA level in liver metastases compared to primary tumors

Table I. Range, standard deviation, and means of fluorescent intensity of MET and HGF staining in neoplastic and non-neoplastic tissues.

	MET			HGF		
	Range	SD	Mean	Range	SD	Mean
Normal (surrounding carcinoma)	43.6-162.1	31.15	94.30	26.6-63.3	8.70	34.20
Tumor (carcinoma)	47.5-196.3	38.16	111.21	22.8-65.4	8.40	32.90
Tumor:normal ratio (carcinoma)[a]	-0.8-1.6	0.44	0.19	-1.0-1.2	0.46	-0.08
Normal (surrounding adenoma)	53.2-190.7	39.48	109.50	22.2-48.0	6.40	31.60
Tumor (adenoma)	47.7-197.3	36.03	116.30	23.3-41.7	4.30	30.30
Tumor:normal ratio (adenoma)[a]	-0.7-1.4	0.48	0.16	-0.9-0.7	0.34	-0.05

[a]Ratios were log transformed (base 2) to normalize the ratio distributions.

Table II. Means and standard deviations of fluorescent intensity of MET and HGF staining in tumor tissues and log-transformed tumor:normal ratio of staining intensity within histopathological subgroups.

	MET				HGF			
	Tumor mean	Tumor SD	Log T:N mean	Log T:N SD	Tumor mean	Tumor SD	Log T:N mean	Log T:N SD
Carcinomas								
Grade								
1 and 2	112.8	36.8	0.24	0.42	32.0	8.3	-0.12	0.43
3	105.7	44.2	-0.04	0.45	35.5	8.5	0.08	0.55
pTNM stage								
I	103.3	38.9	0.16	0.28	34.9	9.0	0.11	0.34
II	111.3	39.1	0.05	0.46	32.0	10.3	-0.13	0.60
III	121.3	37.6	0.43	0.61	32.3	5.2	-0.26	0.25
IV	110.2	20.6	0.25	0.31	31.8	6.5	-0.19	0.49
WHO subtypes								
Tubular/papillary	112.8	36.8	0.24	0.42	32.0	8.3	-0.12	0.43
Mucinous	102.4	45.6	-0.04	0.45	35.1	8.7	0.08	0.55
Adenomas								
Low-grade intraepithelial neoplasia	116.6	31.0	0.15	0.44	30.5	4.5	-0.01	0.36
High-grade intraepithelial neoplasia	115.8	46.2	0.18	0.56	29.9	4.22	-0.09	0.32

and normal mucosa, while the extent of protein expression detected immunohistochemically correlated with the mRNA expression (16). Hiscox *et al* reported an overexpression of c-met RNA in all cases (n=21) under study, whereas only 75% displayed up-regulation of the protein (6). On the other hand, Southern blot analysis and RT-PCR revealed an amplification of the c-met gene in 12% of colorectal cancer samples (n=43), and an overexpression in 30% only. Significant associations with clinical parameters and survival were not observed (59). In a previous investigation involving normal epithelium from eight patients and 36 primary colorectal carcinomas, a significantly higher number of c-met mRNA copies were detected in the latter. Furthermore, c-met mRNA copy number correlated with the depth of invasion as well as being enhanced in pN1/pN2 stage patients compared to pN0 patients (46). A published study using tissue microarray analysis of 110 colorectal tissues by phosphor image analysis after c-met mRNA *in situ* hybridisation showed a 2.5-fold increase in carcinomas compared to normal tissues (60).

Other studies have measured MET protein by Western blotting and immunohistochemistry. Paraffin-embedded tumor samples from 86 patients with colon cancers in Dukes' B and C stages indicated a significant up-regulation of the MET protein in stage C vs. stage B carcinomas (44). In an immunohistochemical investigation on frozen tissue sections of normal mucosa (n=6), colorectal adenomas (n=6) and carcinomas (n=54) an increase of semi-quantitatively evaluated MET protein expression along the adenoma-carcinoma sequence was reported (47). In the same study, an enhancement of HGF mRNA expression in carcinomas vs. normal tissues was additionally observed, and HGF protein expression was detected in cells present within the tumor stroma. In a prognostic study using tissue microarrays, an association between MET expression and survival probability in TNM stage II patients was not observed (61).

In the present study, we utilized an immunohistochemical double-staining method in order to co-localize MET and HGF in normal and neoplastic colorectal tissues. By immunofluorescent double-labelling, both molecules were detected and quantified by confocal laser microscopy. According to our data, and in contrast to numerous studies measuring c-*met* mRNA, expression differences between normal mucosa, adenomas and carcinomas were not significant. Analogously, the grade of intraepithelial neoplasia of adenomas as well as the histopathological subtype, pTNM staging and grading of carcinomas did not correlate with differences regarding the MET protein levels. HGF did not exhibit significant associations with these clinico-pathological parameters. However, the ratio of HGF expression in tumor vs. non-neoplastic epithelia is a predictive parameter for high vs. low pTNM stage.

Our data result from the first quantitative evaluation of MET and HGF proteins in the colorectal adenoma-carcinoma sequence, for which previous studies reported contradictory results (44,47). When interpreting these data, several aspects regarding HGF-MET interactions are of interest. Whereas MET was expressed in cells of epithelial origin, HGF expression was mostly confined to mesenchymal cells suggesting paracrine effects on the tumor cells (16), as confirmed by our results. On the other hand, HGF bioactivity may be controlled by tumor-cell derived factors such as HGF activator (HGFA) and its inhibitor HGFA inhibitor type 1 (HAI-1), which are both detected in colorectal mucosa as well as neoplasms (45).

Therefore, it is tempting to speculate that HGF-MET interactions may depend on various other factors involved in their regulation.

With regard to previous data describing an overexpression of c-*met* mRNA, the turnover of MET protein in colorectal neoplasms should be included in further studies. In addition, in other organ systems the level of MET protein expression also exhibits differences between normal epithelia and their respective neoplasms. Applying the same immunofluorescent staining and quantitative analysis by confocal laser microscopy as in the present study, ovarian neoplasms exhibited a stronger staining compared to ovarian surface epithelium (51), whereas nasopharyngeal carcinomas showed an intermediate MET protein level as compared with either squamous cell or respiratory epithelium (53). Furthermore, the degree of MET tyrosine phosphorylation may represent a decisive factor in this context, since highly metastatic colorectal carcinoma cell lines expressed a constitutively tyrosine phosphorylated MET, whereas cell lines with a low metastatic potential did not (62). In colorectal cancer cell subclones with an only modestly reduced MET expression, a 60-90% reduction in basal MET autophosphorylation and kinase activity correlated with significantly reduced *in vitro* and *in vivo* growth rates of these cells (63). Our data and these observations indicate that functional aspects and not only the level of protein expression determine the biological role of HGF-MET interactions during colorectal carcinogenesis. Furthermore, interactions of MET with tyrosine-phosphorylated ß-catenin may exert influences on T cell factor (tcf) activity and transcriptional target genes such as cyclin D1, c-*myc* and uPAR (62). The significance of HGF expression in this analysis of colonic tumors and adenomas also suggests that an adjuvant therapy either as an antibody or ligand analogue that inactivates or targets the Met receptor or neutralizes the natural ligand HGF/SF could be of therapeutic benefit.

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