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Expression of c-Met and Heparan-Sulfate Proteoglycan Forms of CD44 in Colorectal Cancer

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In colorectal cancer patients, prognosis is not determined by the primary tumor but by the formation of distant metastases. Molecules that have been implicated in the metastatic process are the proto-oncogene product c-Met and CD44 glycoproteins. Recently, we obtained evidence for functional collaboration between these two molecules: CD44 isoforms decorated with heparan sulfate chains (CD44-HS) can bind the c-Met ligand, the growth and motility factor hepatocyte growth factor/scatter factor (HGF/SF). This interaction strongly promotes signaling through the receptor tyrosine kinase c-Met. In the present study, we explored the expression of CD44-HS, c-Met, and HGF/SF in the normal human colon mucosa, and in colorectal adenomas and carcinomas, as well as their interaction in colorectal cancer cell lines. Compared to the normal colon, CD44v3 isoforms, which contain a site for HS attachment, and c-Met, were both overexpressed on the neoplastic epithelium of colorectal adenomas and on most carcinomas. Likewise, HGF/SF was expressed at increased levels in tumor tissue. On all tested colorectal cancer cell lines CD44v3 and c-Met were co-expressed. As was shown by immunoprecipitation and Western blotting, CD44 on these cells lines was decorated with HS. Interaction with HS moieties on colorectal carcinoma (HT29) cells promoted HGF/SF-induced activation of c-Met and of the Ras-MAP kinase pathway. Interestingly, survival analysis showed that CD44-HS expression predicts unfavorable prognosis in patients with invasive colorectal carcinomas. Taken together, our findings indicate that CD44-HS, c-Met, and HGF/SF are simultaneously overexpressed in colorectal cancer and that HS moieties promote c-Met signaling in colon carcinoma cells. These observations suggest that collaboration between CD44-HS and the c-Met signaling pathway may play an important role in colorectal tumorigenesis. *(Am J Pathol 2000, 157:1563–1573)*

Colorectal cancer evolves through a series of morphologically recognizable stages known as the adenoma-carcinoma sequence.[1] Primarily as a result of this stepwise development, the molecular genetics of colorectal cancer are among the best studied of any solid neoplasm,[2–5] and serve as a paradigm for multistep tumorigenesis. Several important molecules implicated in the tumorigenetic process act on the cell cycle, resulting in a disturbed homeostasis between cell proliferation and apoptosis.[2] The main cause of tumor-related death in colorectal cancer however, is the formation of distant metastases, rather than the growth of the primary tumor. Although relatively little is known concerning the molecular mechanisms underlying this complex process, recent studies have identified CD44 glycoproteins[6] and the c-Met receptor tyrosine kinase[7,8] as potentially important components of the metastatic cascade.

CD44 is a family of transmembrane receptors generated from a single gene by alternative splicing and differential glycosylation.[9–13] Important biological processes involving CD44 glycoproteins include cell adhesion,[14] lymphocyte homing,[9,15,16] hematopoiesis,[9] and tumor progression and metastasis.[6,9,11,17,18] In colorectal cancer, CD44 glycoproteins, which are normally detected only in the lower crypt epithelium of the intestinal mucosa, are overexpressed.[6,19–24] This overexpression is an early event in the colorectal adenoma-carcinoma sequence[25,26] suggesting a causal relation to loss of *APC* tumor suppressor gene function. Indeed, recent studies in *Apc* and *Tcf-4* mutant mice indicate that CD44 expression in normal and neoplastic intestinal epithelium is regulated by the Wnt-signaling pathway.[24]

The precise mechanisms via which CD44 promotes tumorigenesis have not yet been elucidated. CD44 functions as a molecular linker between extracellular matrix molecules, specifically hyaluronate, and the cell and cytoskeleton.[9,14,27,28] Recently, CD44 isoforms decorated with heparan sulfate-side (HS) chains have been shown to bind and present growth factors.[29–31] We demonstrated that CD44-HS binds the growth and motility factor hepatocyte growth factor/scatter factor (HGF/SF). This interaction strongly promotes signaling through c-Met,

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the high-affinity receptor for HGF/SF.[31] The HGF/SF-c-Met pathway is essential for normal murine embryonal development[32–34] and affects a wide range of biological activities including angiogenesis, cell motility, growth, and morphogenesis. In addition, there is ample evidence for a key role of the HGF/SF-c-Met pathway in tumor growth, invasion, and metastasis.[7,8,35,36] For example, c-Met was isolated originally as the product of a human oncogene, Tpr-Met, which encodes a constitutively dimerized/activated chimeric c-Met protein possessing transforming activity.[37,38] The generation of an autocrine loop as a result of co-expression of wild-type c-Met and HGF/SF molecules in the same cell is also oncogenic.[39] The tumorigenicity of both Tpr-Met and autocrine HGF/SF-Met signaling has been verified in transgenic mouse models, which develop tumors in many different tissues including mammary glands, skeletal muscles, and melanocytes.[40,41] c-Met activation has also been shown to promote the metastatic spread of cancer, a finding that likely is because of its stimulatory effects on a variety of processes such as angiogenesis, cell motility, and protease secretion.[8,42] Recently, missense mutations in c-Met were found to be associated with human papillary renal carcinomas.[43] These mutations deregulate the enzymatic activity of the receptor, thereby unleashing its oncogenic potential.[44]

To explore whether collaboration between CD44-HS and the HGF/SF-c-Met pathway is an option in colorectal cancer, the present study investigates the expression of these molecules in the normal colon mucosa as well as along the distinct steps of the colorectal adenoma-carcinoma sequence.

Materials and Methods

Antibodies

Mouse monoclonal antibodies (mAbs) used were: 3G5 (anti-CD44v3, IgG2b; R&D Systems, Abington, UK); 3G10 (anti-desaturated uronate from heparitinase-treated HS; HS stub, IgG2b)[45]; PY-20 (anti-phosphotyrosine, IgG2b; Affiniti, Nottingham, UK); anti-human c-Met (IgG2a; R&D Systems); anti-human HGF/SF (IgG1; R&D Systems). Polyclonal antibodies used were (rabbit anti-c-Met, IgG, C12; Santa Cruz Biotechnology, Santa Cruz, CA); rabbit anti-phospho-p44/42 MAP kinase (Thr202/Tyr204; New England Biolabs, Beverly, MA); rabbit anti-ERK 1 (C-16; Santa Cruz Biotechnology); horseradish-peroxidase (HRP)-conjugated rabbit anti-mouse (DAKO, Glostrup, Denmark); HRP-conjugated goat anti-rabbit (DAKO); HRP-conjugated swine anti-rabbit (DAKO); biotin-conjugated rabbit anti-mouse (DAKO). In addition we used phycoerythrin-conjugated streptavidin (DAKO).

Cell Lines

The colon carcinoma cell lines SW480, SW620, colo 201, colo 205, colo 320, and HT-29, were purchased from the American Type Culture Collection (ATCC, Rockville, MD). HT-29 cells were cultured in modified McCoy's 5A medium (Gibco BRL/Life Technologies, Paisley, UK), whereas SW480 and SW620 were cultured in L-15 (Leibovitz) medium (Gibco BRL/Life Technologies). The other cell lines were cultured in RPMI 1640 (Gibco BRL/Life Technologies). All media were supplemented with 10% heat-inactivated fetal calf serum, 2 mmol/L L-glutamine, 100 IU/ml penicillin, and 100 IU/ml streptomycin (all from Gibco BRL/Life Technologies).

Purification of Wild-Type and Mutant HGF/SF

The construction of pVL1393 vectors (Pharmingen, San Diego, CA) containing wild-type or mutant HGF/SF (HP1) cDNA was described elsewhere.[46] HGF/SF (wild type and HP1) was produced in a Baculovirus system as described previously.[47] In brief, sf 9 insect cells were transduced with an amplified virus stock and after 3 days media were pooled and analyzed for scattering activity in the Madin-Darby canine kidney dissociation assay.[48] Then, HGF/SF was purified with Ni-NTA-resin from the QIA expressionist system (Qiagen, Hilden, Germany). HGF/SF concentrations were measured by enzyme-linked immunosorbent assay as described previously,[49] and in addition, HGF/SF (wild type and mutant) was analyzed by Western blotting using anti-goat-HGF/SF.

Enzyme Treatments

For enzymatic cleavage of GAGs, cells were treated with either heparitinase (*Flafobacterium heparinum*, EC 4.2.2.8; ICN Biomedicals, Aurora, OH) or chondroitinase avidin-biotin-peroxidase complex (*Proteus vulgaris*, EC 4.2.2.4; Boehringer Mannheim, Almere, The Netherlands) in phosphate-buffered saline (PBS) at 37°C for the periods indicated. Enzyme treatments were followed by immunoprecipitation.

Immunoprecipitation and Western Blot Analysis

Immunoprecipitation was performed as described.[49] The only modification were that, for precipitation of CD44, cells were lysed in lysis buffer containing 50 mmol/L Tris-HCl, pH 8, 150 mmol/L NaCl, 1% Nonidet P-40, 10 μg/ml aprotinin (Sigma), 10 μg/ml leupeptin (Sigma), 1 mmol/L sodium orthovanadate (Sigma), 2 mmol/L ethylenediaminetetraacetic acid, and 5 mmol/L NaF. For precipitation of c-Met, cells were lysed in 10 mmol/L Tris-HCl (pH 8), 150 mmol/L NaCl, 10% glycerol, 1% Nonidet P-40, 10 μg/ml aprotinin (Sigma), 10 μg/ml leupeptin (Sigma), 2 mmol/L sodium orthovanadate (Sigma), 5 mmol/L ethylenediaminetetraacetic acid, and 5 mmol/L NaF.

Western blotting of immunoprecipitates and total cell lysates was essentially performed as described previously,[50] with the modification that, for analysis of phosphorylated proteins, membranes were blocked and stained in 2% bovine serum albumin, 20 mmol/L Tris-HCl, 150 mmol/L NaCl, pH 7.5, and 0.05% Tween-20 (Sigma). Films were scanned with an Eagle Eye II video system (Stratagene, La Jolla, CA) and band intensities were determined with ONE-Dscan software (Stratagene). c-Met

phosphorylation was expressed as the ratio of phosphorylated c-Met to precipitated c-Met.

Activation of the MAP kinases ERK 1 and 2 was analyzed by immunoblotting of total cell lysates with the phospho-specific p44/42 MAP kinase antibody.

Tissue Samples

The study set consisted of 54 primary colorectal carcinomas,[20] removed at operation between January 1, 1983 and January 1, 1986 at the Department of Surgery, Reinier de Graaf Hospital, Delft, The Netherlands, of which snap-frozen tissue and follow-up till June 1, 1992 (6.5 to 9.5 years) was available. The mean age of the patients at diagnosis was 69.7 (range, 39 to 92) and the male-to-female ratio was 28/40. Colorectal tissue samples of six adenomas and six normal controls, removed at operation between January 1, 1992 and January 1, 1999 were obtained from the tissue bank of the Department of Pathology, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands.

Immunohistochemistry and Statistical Analysis

Frozen tissue sections were tested for the expression of CD44v3, c-Met, and HGF/SF by immunohistochemistry as described previously.[18,19] A single modification was that HRP-conjugated rabbit anti-mouse and HRP-conjugated swine anti-rabbit were used as secondary and tertiary antibodies, respectively. All slides were read by two independent observers, and discrepancies were solved by consensus. The tumor samples were scored as described previously:[20,23] 0 (low/negative) = <10% of the cells positive; 1 (intermediate) = 10 to 50% of the cells positive; 2 (high) = >50% of the cells positive. Survival functions were estimated by the Kaplan-Meier method and comparison of survival functions between groups was performed by the log-rank test.

RNA Isolation and Reverse-Transcriptase-Polymerase Chain Reaction (RT-PCR)

RNA isolation and first-strand cDNA synthesis were performed as described previously.[49] PCR was performed with 1.5 U *Taq* DNA Polymerase (Gibco BRL/Life Technologies), 200 μmol/L dNTPs (Pharmacia Biotech, Uppsala, Sweden), and 1.5 mmol/L $MgCl_2$ (2 mmol/L for GAPDH) in 1× PCR Buffer (both Gibco BRL/Life Technologies). Primers used were HGF-1 (5′-CGACAGTGTTTCCCTTCTCG-3′) in combination with HGF-3 (5′-GGTGGGTGCAGACACAC-3′), or GAPDH-D (5′-GGCAGAGATGATGACCCTTTTGGC-3′) in combination with GAPDH-U (5′-AAGGTGAAGGTCG-GAGTCAACG-3′). PCR was started with a 5-minute denaturation step at 95°C, after which amplification was performed in 35 cycles of denaturation at 95°C for 30 seconds, annealing at 60°C (55°C for GAPDH) for 1 minute (30 seconds for GAPDH), and elongation at 72°C for 2 minutes (30 seconds for GAPDH). After a final elongation step for 10 minutes at 72°C, samples were cooled on ice and analyzed by electrophoresis in an 1.5% agarose Tris borate-ethylenediaminetetraacetic acid-gel containing ethidium bromide.

Fluorescence-Activated Cell Sorting Analysis (FACS)

For FACS analysis cells were blocked with 10% pooled human serum (CLB, Amsterdam, The Netherlands), 1% bovine serum albumin (Fraction V) (Sigma) in PBS at 4°C for 15 minutes, and washed with FACS buffer (1% bovine serum albumin in PBS), respectively. Then, the cells were incubated with the primary antibodies for 1 hour, washed, and incubated with the secondary antibody for 30 minutes. All incubations were performed in FACS buffer at 4°C. Stained cells were analyzed by flow cytometry on a FACScan (Becton Dickinson, Mountain View, CA).

HS Proteoglycan-Dependent Phosphorylation of c-Met in HT29 Cells

HT29 cells were cultured in 6-well plates until subconfluent (60 to 80%) and then starved overnight. Part of the cells were treated with heparitinase as mentioned above and activated with 100 ng/ml wild-type HGF/SF or mutant HGF/SF (HP1) in 0.5 ml serum-free, prewarmed medium for 10 minutes at 37°C. Cells were washed once with cold PBS and were immediately cooled on ice. Lysis buffer (500 μl) was added and cells were harvested. c-Met was immunoprecipitated and c-Met phosphorylation was analyzed by Western blotting.

Results

Co-Expression of CD44-HS and c-Met in Colorectal Cancer

Previous studies have shown that heparan sulfate forms of CD44 (CD44-HS) are splice variants containing exon v3.[13,31] To explore the expression of CD44-HS during colorectal tumor progression, we compared CD44v3 levels in normal colon mucosa, adenomas, and carcinomas (Table 1 and Figure 1). In the normal colon mucosa CD44v3 expression was low to intermediate and strictly confined to the base of the crypts. By contrast, in all adenomas and in 70% (38 of 54) of the invasive carcinomas, an intermediate to high expression of CD44v3 was observed (Table 1).

For c-Met, enhanced expression along the adenoma-carcinoma sequence was also observed. Whereas the epithelium of the normal colon mucosa showed a low to intermediate expression, c-Met expression in adenomas and carcinomas was intermediate to high and high, respectively (Table 1; Figure 1).

Table 1. Expression of CD44v3 and c-Met in Colorectal Tumorigenesis

Tissue	Expression level*	Number of patients *n* (%)	
		CD44v3	c-Met
Normal			
Crypt base	Negative/low	1 (17)	3 (60)
	Intermediate	4 (67)	2 (40)
	High	1 (17)	0
Upper crypt	Negative/low	6 (100)	3 (60)
	Intermediate	0	2 (40)
	High	0	0
Polyp	Negative/low	0	0
	Intermediate	4 (67)	2 (33)
	High	2 (33)	4 (67)
Carcinoma	Negative/low	16 (30)	0
	Intermediate	19 (35)	0
	High	19 (35)	54 (100)

*Negative/low, intermediate, high: expression on <10%, 10 to 50%; or >50% of the tumor cells, respectively.

Colorectal Carcinoma Cell Lines Co-Express CD44v3 and c-Met

To strengthen the observation that colorectal carcinomas co-express CD44v3 and c-Met, the presence of these molecules was assessed by FACS on a panel of colorectal carcinoma cell lines. On all of these carcinoma cell lines, ie, colo 320, HT29, SW480, SW620, colo 201, colo 205, and colo320, both CD44v3 and c-Met were clearly expressed (Figure 2).

Taken together, the above expression studies show that co-expression of CD44v3 and c-Met is present in most primary colorectal adenomas and carcinomas as well as in colorectal carcinoma cell lines.

CD44v3 on Colorectal Cells is Decorated with HS

To verify whether the glycosylation machinery of colorectal cancer cells indeed decorates CD44v3 with HS chains, we studied CD44v3 immunoprecipitates from the colorectal cancer cell lines SW480 and HT-29 on Western blot with the mAb 3G10. This mAb recognizes the HS

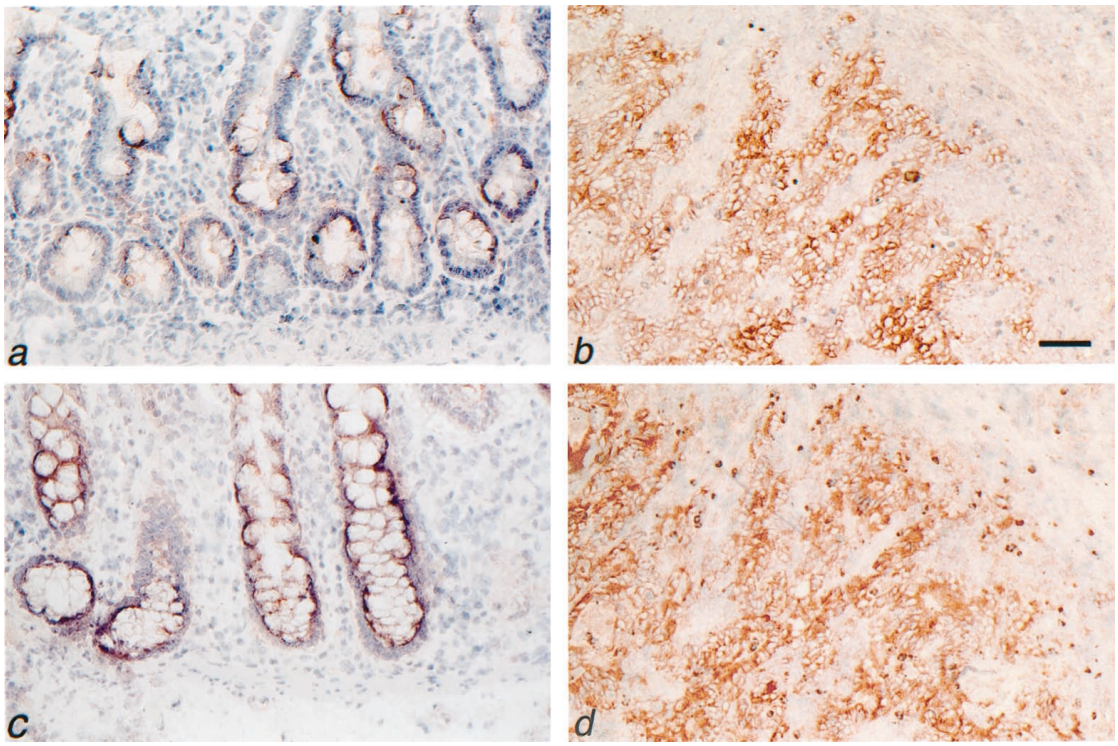

Figure 1. Expression of CD44v3 and c-Met by normal colon mucosa and colorectal carcinomas. Normal colon mucosa (**a** and **c**) and colorectal carcinoma (**b** and **d**) serial frozen tissue sections were stained for CD44v3 (**a** and **b**) or for c-Met (**c** and **d**) by immunohistochemistry. **a:** Normal colon mucosa showing weak focal expression of CD44v3 in the lower part of the crypts. **b:** Invasive colorectal carcinoma with strong CD44v3 expression. **c:** Normal colon mucosa showing weak expression of c-Met. **d:** Invasive colorectal carcinoma with strong c-Met expression. Tissues were counterstained with hematoxylin. Scale bars, 57 μm (**a** and **c**); 69 μm (**b** and **d**).

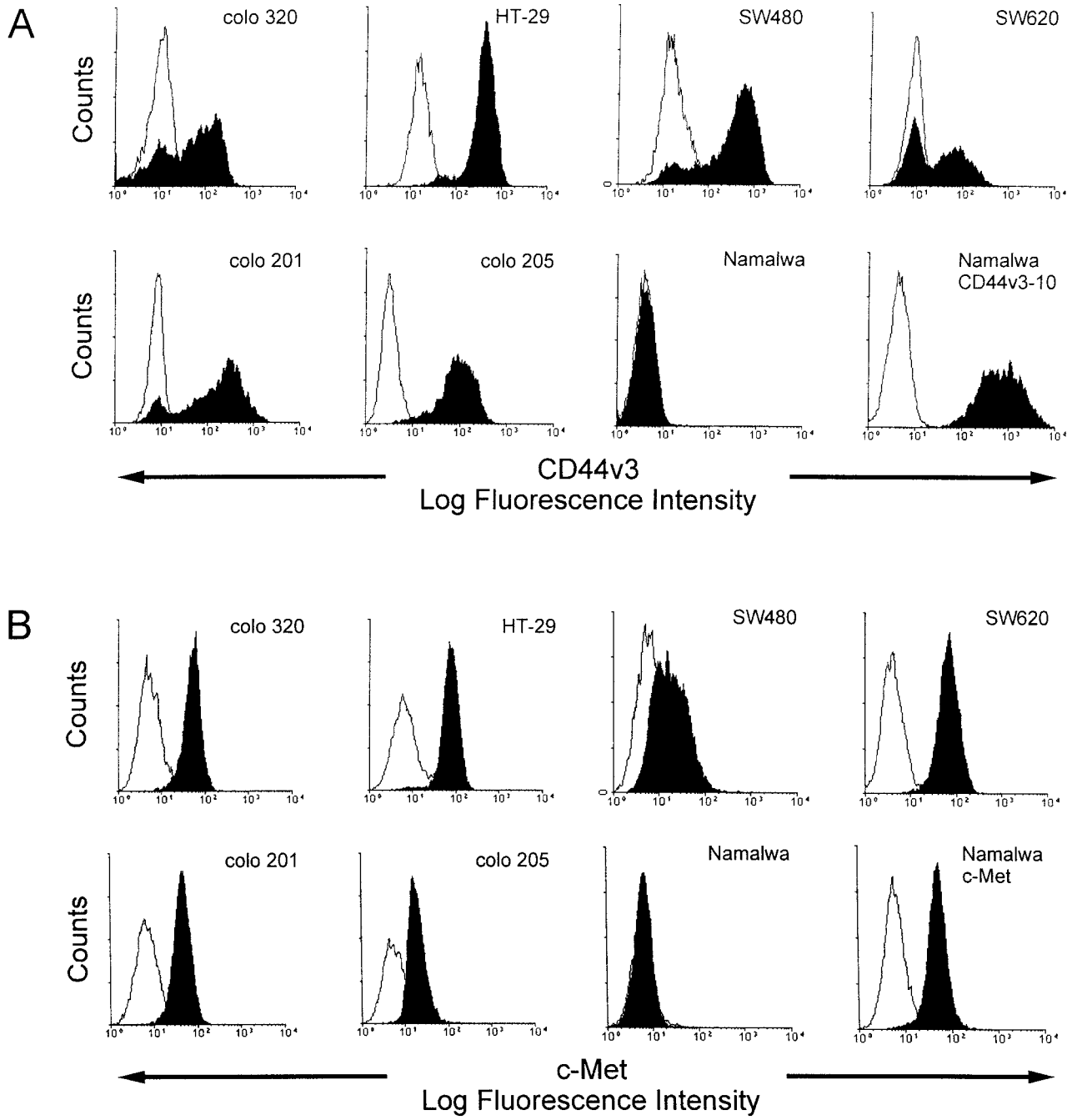

Figure 2. Expression of CD44v3 and c-Met by colon carcinoma cell lines. **A:** FACS analysis of the expression of CD44v3 on the colon carcinoma cell lines colo 320, HT29, SW480, SW620, colo 201, colo 205, and colo 320. Wild-type and CD44v3–10 transfected Namalwa cells are shown as negative or positive controls, respectively. Expression was analyzed with mouse anti-CD44v3 (**filled histogram**) or an isotype-matched control antibody (**empty histogram**), followed by RPE-conjugated goat anti-mouse. **B:** FACS analysis of the c-Met expression on the colon carcinoma cell lines shown in **A**. Wild-type or c-Met-transfected Namalwa cells are shown as negative and positive controls, respectively. Expression was analyzed with mouse anti-c-Met (**filled histogram**) or an isotype-matched control antibody (**empty histogram**), followed by RPE-conjugated goat anti-mouse.

stubs that remain on HS proteoglycans after heparitinase treatment.[45] Hence, before immunoprecipitation, the tumor cells were treated with heparitinase, or as controls, were sham-treated or chondroitinase-treated. As is shown by staining with an anti-pan CD44 mAb (Figure 3), one major CD44v3 species of ~200 kd was precipitated from SW480 cells whereas two species of ~150 and 200 kd were precipitated from HT-29. The size of the latter CD44 variant was identical to that from a control cell line (Namalwa) expressing a single CD44 isoform containing v3-10,[31,51] whereas the shorter species most probably contains a shorter variable domain. Staining of the blots with the anti-HS stub mAb revealed the presence of bands corresponding to those obtained after staining with the anti-pan CD44 mAb. These bands were specifically present in the precipitates of the heparitinase

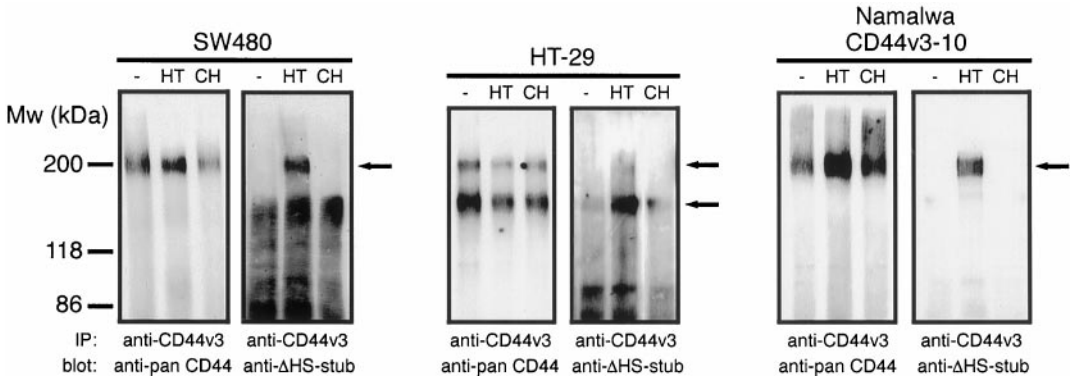

Figure 3. CD44v3 isoforms on colon carcinoma cell lines are decorated with HS. CD44v3 was immunoprecipitated from the colon carcinoma cell lines SW480 and HT-29, and, as a positive control, from Namalwa cells transfected with CD44v3–10, with mouse anti-CD44v3. Before immunoprecipitation, the cells were treated with either PBS (−), 30 mU/ml heparitinase (HT), or 30 mU/ml chondroitinase ABC (CH) at 37°C for 3.5 hours. The Western blot of the precipitates was stained with the anti-pan CD44 mAb Hermes-3, stripped, and restained with the mAb 3G10 that detects ΔHS stubs after treatment of HS with heparitinase. CD44v3 isoforms decorated with HS are indicated with **arrows**.

treated cells, but not in the precipitates of sham- or chondroitinase-treated cells (Figure 3). Hence, CD44v3 isoforms on colorectal cancer cell lines are HS-decorated.

Expression of HGF/SF in Colorectal Cancer Tissue Samples

To explore whether the c-Met ligand HGF/SF is also expressed within the colorectal carcinoma microenvironment, HGF/SF mRNA expression was measured by RT-PCR in paired samples of normal and neoplastic colon tissue from five patients. In addition, tissue sections of normal colon mucosa and carcinomas were stained for the presence of HGF/SF protein. As is shown in Figure 4A, HGF/SF mRNA expression was readily detectable in all colorectal carcinoma samples. Moreover, the intensity of the bands obtained from the tumor samples was clearly increased, compared to those obtained from the samples of the normal mucosa. In colorectal carcinomas HGF/SF protein expression was detected in cells present within the tumor stroma (Figure 4B). In normal mucosa no HGF/SF-positive cells were found (data not shown).

HS on Colorectal Cancer Cells Promotes Ligand-Induced c-Met Phosphorylation

To investigate whether HS chains on colorectal cancer cells are able to present HGF/SF to c-Met and promote signaling, the tyrosine phosphorylation of c-Met was studied in HT-29 cells that: 1) were treated with heparitinase before HGF/SF stimulation; 2) were stimulated with HP1, a non-HS-binding mutant form of HGF/SF.[46] As is shown in Figure 5A (top), heparitinase treatment of HT-29 cells led to an almost complete reduction in the HGF/SF-induced phosphorylation of c-Met. Similarly, the HGF/SF mutant HP1 was significantly less potent in inducing c-Met phosphorylation (Figure 5B). In addition to activation of c-Met, the HGF-induced activation of the MAP kinases ERK 1 and 2 was shown to be dependent on the presence of HS moieties as well (Figure 5A, bottom). These findings indicate that interaction of HGF/SF with HS proteoglycans expressed on the surface of colorectal cancer cells facilitates c-Met signaling.

CD44v3 Expression Is Related to Poor Prognosis

Previous studies from our own and other laboratories have shown that CD44 splice variants containing v6 and v8-10 are unfavorable prognosticators in colorectal cancer. Expression of these variants on the primary tumor predicts metastatic disease and tumor-related death.[20–23] In view of the ability of CD44v3 to present growth factors, which may promote metastasis, we now studied whether expression of CD44v3 also predicts prognosis. CD44v3 was assessed in a study group of 54 colorectal cancer patients with a long-term (6.5 to 9.5 years) and complete follow-up. Details on this study group have been published previously.[20,23] As is depicted in Table 2 and Figure 6, CD44v3 expression on the primary tumor indeed predicts tumor related death. CD44v3 expression on the tumors was strongly correlated to expression of CD44v6 (data not shown).

Discussion

Deregulation of c-Met signaling can initiate and promote tumor growth and dissemination.[7,8,35–39] The present study shows that c-Met is strongly expressed on primary colorectal adenomas and carcinomas, as well as on colorectal cell lines, whereas HGF/SF is present at increased levels within tumor tissue. In addition, it demonstrates that a subset of colorectal carcinomas with unfavorable prognosis strongly expresses CD44-HS. Because CD44-HS can bind and present HGF/SF, and promotes signaling through c-Met,[31] our observations suggest a role for functional collaboration between CD44-HS and the HGF/SF-c-Met pathway in colorectal tumorigenesis.

Our observation that c-Met is strongly expressed by both colorectal adenomas and carcinomas (Table 1 and Figure 1) confirms previous studies, documenting over-

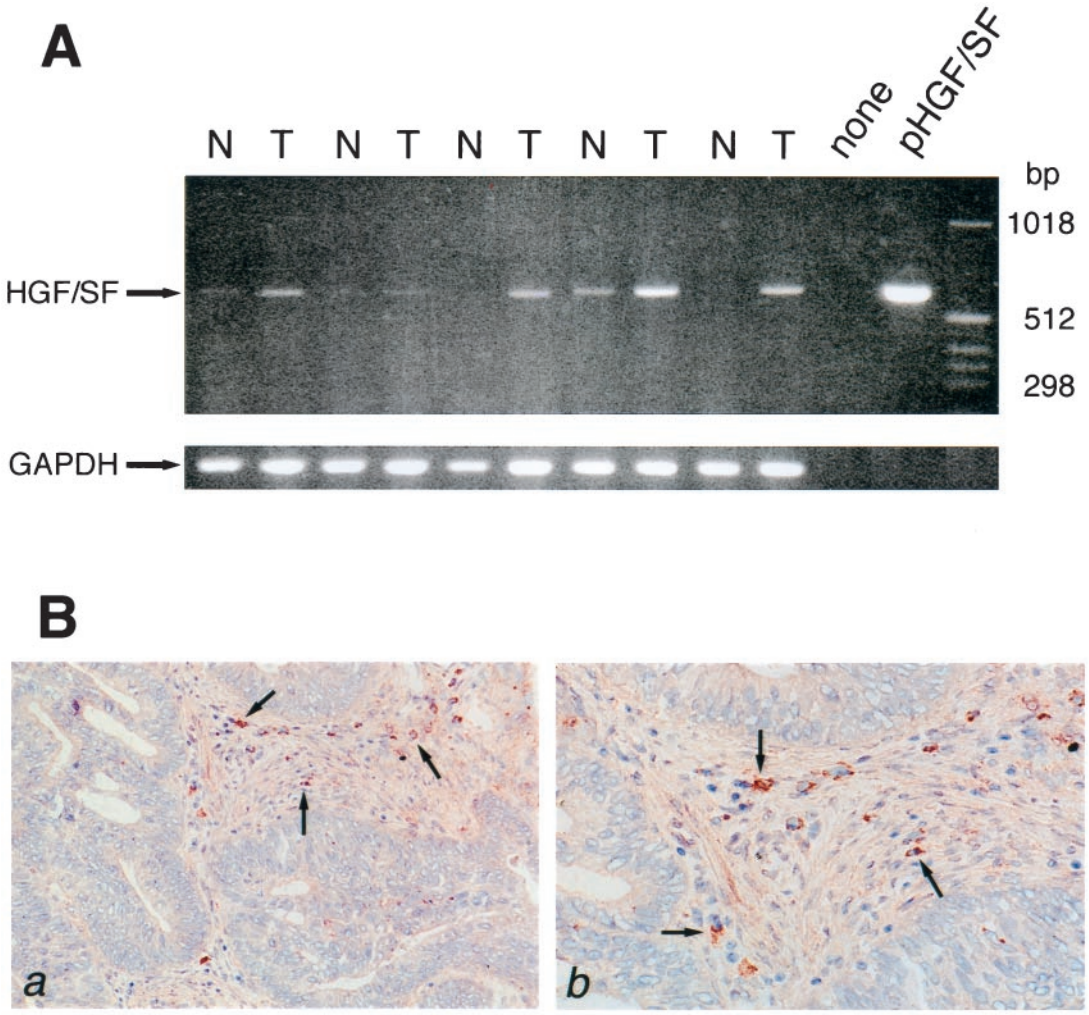

Figure 4. HGF/SF mRNA and protein expression in normal colon mucosa and colorectal carcinomas. **A:** RT-PCR was performed on total RNA isolated from five pairs of normal colon (N) and primary colorectal carcinoma (T), on water (none), and on a plasmid containing full-length human HGF/SF cDNA (pHGF/SF). Primers used were HGF/SF-specific or, as a control, glyceraldehydephosphate dehydrogenase (GAPDH)-specific. The tumors analyzed were CD44- and Met-positive. **B:** HGF/SF protein expression in colorectal cancer was assessed by immunohistochemistry. **a** and **b:** Frozen sections from colorectal cancer tissue were stained with anti-human HGF/SF. This identified cells (**arrows**) in the tumor stroma as HGF/SF-producing cells.

expression of c-Met in colorectal tumors.[52–54] We extend these findings by demonstrating that, in parallel, HGF/SF is expressed in colorectal tumor tissue. HGF/SF mRNA levels in tumor tissue were consistently higher than in the normal mucosa (Figure 4A). Moreover, cells expressing HGF/SF protein were detected in the stroma of tumors but not in the normal mucosa (Figure 4B). These observations indicate that paracrine HGF/SF-c-Met interaction is likely to take place within the colorectal carcinoma microenvironment, promoting tumor growth and motility.

At present, the mechanism of c-Met and HGF/SF overexpression in colorectal cancer is primarily unknown. Di Renzo and colleagues[53] reported that c-Met overexpression is associated with amplification of the c-*met* gene in ~10% of primary colon carcinomas and 50% of metastases. However, because high c-Met levels were present in all carcinomas tested (Table 1) other mechanisms must also be involved. As c-Met overexpression occurs from an early stage of colorectal tumor progression onwards, c-*met* might, like c-*myc*,[55] be regulated by the Wnt-signaling pathway. For HGF/SF the mechanism of overexpression and the identity of cell(s) producing the growth factor within the tumor microenvironment remains to be defined. HGF/SF expression by epithelial tumor cells, with autocrine c-Met stimulation, has been reported in human breast cancer.[56,57] Alternatively, as indicated by our immunohistochemical stainings (Figure 4B), cells within tumor stroma present a potential paracrine source of HGF/SF. Although these cells need further identification, they presumably represent fibroblasts and/or activated macrophages, because both of these cell types have been reported to express HGF/SF.[58–60] Paracrine stimulation may also promote the outgrowth of metastases because HGF/SF is produced at the two major sites of colorectal carcinoma metastasis, ie, the liver[61] and lymphoid tissue.[49]

CD44v3 isoforms were detected on colorectal adenomas, on a major subset (70%) of invasive colorectal carcinomas, and on all carcinoma cell lines studied (Table 1 and Figures 1 and 2). Analyses of CD44v3 immu-

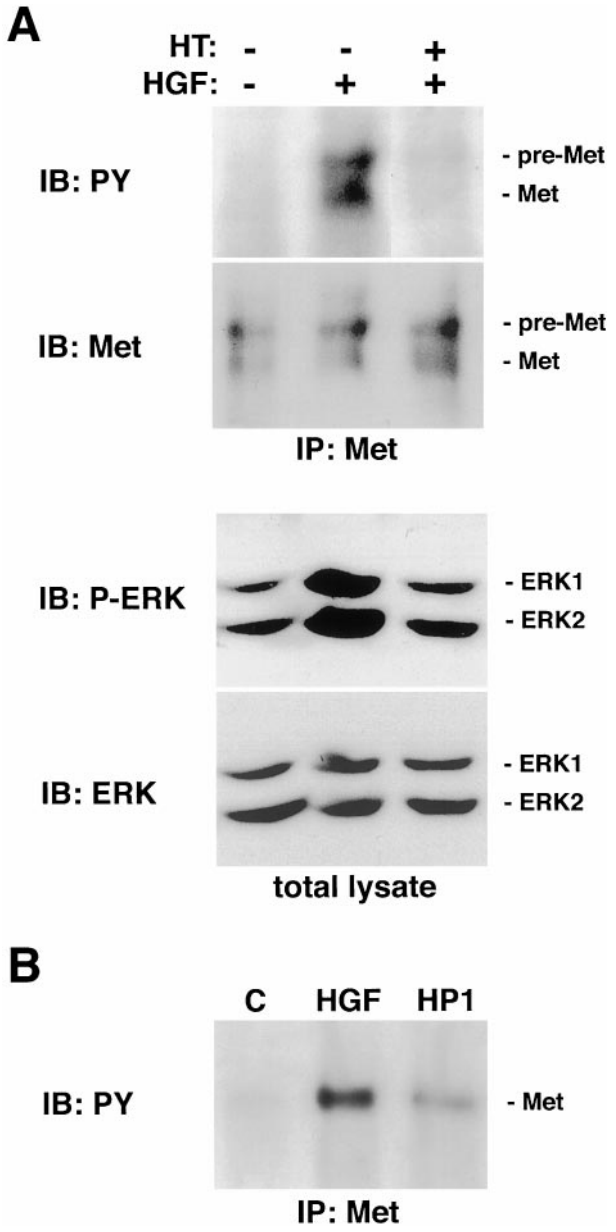

Figure 5. The interaction of HGF with HS moieties of HS proteoglycans promotes Met signaling in HT29 cells. **A:** The effect of heparitinase treatment on HGF-induced Met signaling. HT29 cells were pretreated with 10 mU/ml heparitinase (HT) for 3.5 hours and subsequently stimulated with 100 ng/ml HGF for 10 minutes, as indicated. Met autophosphorylation was analyzed by immunoprecipitation (IP) of Met and immunoblotting (IB) with anti-phosphotyrosine (PY) antibody, and subsequent reprobing of the blot with anti-Met antibody (**top**). In addition, activation of the MAP kinases ERK1 (p44) and 2 (p42) was analyzed by immunoblotting total cell lysates with anti-phospho-ERK1/2 (P-ERK), and subsequent reprobing of the blot with anti-ERK antibody (**bottom**). **B:** Stimulation of Met autophosphorylation by wild-type HGF or a non-HS-binding HGF mutant. HT29 cells were stimulated for 10 minutes with either 100 ng/ml HGF or HP1, a non-HS-binding mutant form of HGF, as indicated, and Met autophosphorylation was analyzed by immunoprecipitation of Met and immunoblotting with anti-phosphotyrosine antibody.

noprecipitates showed that these isoforms were decorated with HS, and thus are HS proteoglycans (Figure 3). Interestingly, interaction of HGF/SF with HS moieties on HT-29 cells was found to promote c-Met phosphorylation as well as activation of the MAP kinases ERK1 and 2

Table 2. Prognostic Significance of CD44v3 Expression

Level of CD44v3 expression*	Number of patients *n* (%)	Median survival in days	*P* value†
Negative/low	16 (30)	1577	
Intermediate	19 (35)	1167	0.03
High	19 (35)	294	

*Negative/low, intermediate, high: expression on <10%, 10 to 50%, or >50% of the tumor cells, respectively.
†Log-rank test from a univariate analysis.

(Figure 5A). Although the precise contribution of CD44-HS *versus* other HS proteoglycans, such as the syndecans[62] remains to be explored, our findings suggest a role of HS proteoglycans in c-Met signaling in colorectal cancer. We have recently identified CD44-HS as a functional co-receptor for HGF/SF. Binding of HGF/SF to CD44-HS promotes signaling through c-Met leading to phosphorylation of several downstream proteins and of overactivity of the Ras-MAP kinase pathway.[31] The Ras-MAP kinase pathway, which has been implicated in the processes of cell motility and invasion,[63] is also activated by Tpr-Met and by oncogenic c-Met mutants associated with human papillary renal carcinomas.[64] The enhancing effects of CD44-HS on signal transduction were critically dependent on HGF/SF interaction with HS moieties, suggesting that CD44-HS promotes the action of HGF/SF through concentration of HGF/SF on the cell surface and by presenting it to the high-affinity receptor c-Met.[31] Similar mechanisms were proposed for the role of high and low affinity receptors in fibroblast growth factor-2 functioning.[65–71] CD44, c-Met and HGF/SF are also expressed in embryonal tissues, including intestine.[24,72] Presumably, they play a role in mesenchymal-epithelial interactions regulating differenti-

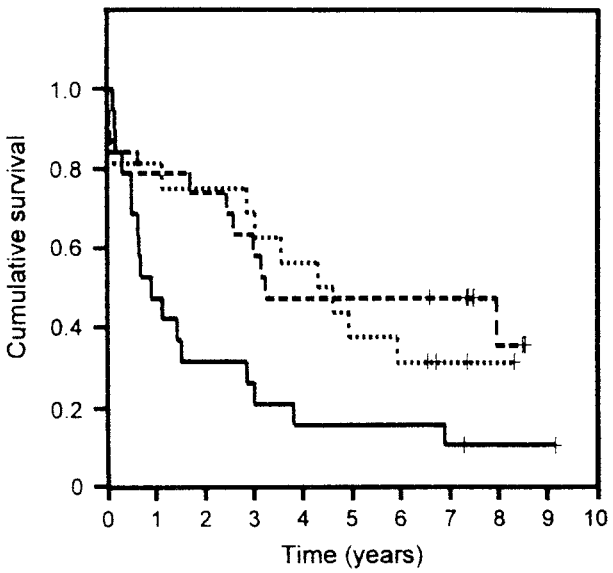

Figure 6. Patient survival and expression of CD44v3. Kaplan-Meier curves showing the relation between the expression of CD44v3 on primary tumors and survival in patients with colorectal carcinoma. **Dotted line**, low expression of CD44v3; **dashed line**, intermediate expression; **solid line**, high expression; +, censored cases.

ation and morphogenesis. Interestingly, we have recently shown that CD44 is present in normal mouse embryonal intestine but absent in that of mice with a disrupted Wnt-signaling pathway.[24] Loss of CD44 in these Tcf-4 mutant mice occurred in the context of a phenotype characterized by the absence of a proliferative stem cell compartment. Binding to CD44-HS of mesenchymally derived growth factors, including HGF/SF and WNT-factors, may be required for normal intestinal stem cell differentiation. In a recent study by Sherman et al,[73] CD44-HS was shown to present fibroblast growth factor-2 in embryonal limb bud formation.

Several studies have reported a strong correlation between CD44 expression in invasive colorectal carcinomas and tumor-related death.[6,20–23,74] In these studies, antibodies recognizing different parts of the CD44 molecule, ie, CD44v6, CD44v8–10, or CD44s (the constant part of CD44) all gave similar results. We now show that CD44v3 expression also predicts prognosis. This correlation of survival with a number of CD44 domains indicates concerted overexpression of these various CD44 variant domains.

In conclusion, we demonstrate that most colorectal tumors co-express c-Met and CD44-HS, and that co-expression of these molecules in invasive carcinomas is associated with an unfavorable prognosis. Moreover, our findings suggest that during colorectal tumorigenesis, CD44-HS overexpression may enhance signaling through the HGF/SF-c-Met signaling pathway, promoting tumor growth and the development of metastatic disease.

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