**Human Cancer Biology**

### The Severity of Neural Invasion Is Associated with Shortened Survival in Colon Cancer


**Abstract**

**Purpose:** Neural invasion (NI) is a histopathologic feature of colon cancer that receives little consideration. Therefore, we conducted a morphologic and functional characterization of NI in colon cancer.

**Experimental Design:** NI was investigated in 673 patients with colon cancer. Localization and severity of NI was determined and related to patient’s prognosis and survival. The neuro-affinity of colon cancer cells (HT29, HCT-116, SW620, and DLD-1) was compared with pancreatic cancer (T3M4 and SU86.86) and rectal cancer cells (CMT-93) in the in vitro three-dimensional (3D)–neural-migration assay and analyzed via live-cell imaging. Immunoreactivity of the neuroplasticity marker GAP-43, and the neurotrophic-chemoattractant factors Artemin and nerve growth factor (NGF), was quantified in colon cancer and pancreatic cancer nerves. Dorsal root ganglia of newborn rats were exposed to supernatants of colon cancer, rectal cancer, and pancreatic cancer cells and neurite density was determined.

**Results:** NI was detected in 210 of 673 patients (31.2%). Although increasing NI severity scores were associated with a significantly poorer survival, presence of NI was not an independent prognostic factor in colon cancer. In the 3D migration assay, colon cancer and rectal cancer cells showed much less neurite-targeted migration when compared with pancreatic cancer cells. Supernatants of pancreatic cancer and rectal cancer cells induced a much higher neurite density than those of colon cancer cells. Accordingly, NGF, Artemin, and GAP-43 were much more pronounced in nerves in pancreatic cancer than in colon cancer.

**Conclusion:** NI is not an independent prognostic factor in colon cancer. The lack of a considerable biologic affinity between colon cancer cells and neurons, the low expression profile of colonic nerves for chemotactic molecules, and the absence of a major neuroplasticity in colon cancer may explain the low prevalence and impact of NI in colon cancer. *Clin Cancer Res; 19(1); 50–61. ©2012 AACR.*

**Introduction**

Neural invasion (NI) is a well-known route of cancer spread in malignant diseases, especially in cancers of the head/neck (1–3), prostate (4, 5), and pancreatic cancer (6–12). Since its initial recognition in the 19th century, the interest in NI has gradually increased, and recent large-scale studies have identified it as a key pathologic feature with a variable impact on patient prognosis (6, 13). As a result of intensifying research on NI, the update of the 7th edition of the tumor–node–metastasis (TNM) classification of malignant tumors has now included perineural invasion as a novel criterion (14). NI prevalence varies considerably among different cancers, with up to 100% in pancreatic cancer, and low frequency in colon cancer (6%–19%; refs. 15, 16). The reasons for the variable NI rates remain unknown. One possibility to explain different NI prevalences among various cancers is the natural organ-innervation of tumor origin. Like every other organ of the gastrointestinal tract, human colon and rectum possess an autonomic extrinsic and intrinsic innervation. The extrinsic innervation is composed of the parasympathetic and sensory fibers from the vagus, and of the sympathetic nerve fibers, which are derived from splanchnic nerves. The intrinsic innervation is represented by the enteric nervous system. Within the colonic wall, the tunica muscularis harbors the myenteric plexus, also known as Auerbach plexus. It is composed of ganglia and nerve fiber strands, creating a finely branched network (17). The muscle layers only contain nerve fiber strands with rarely located singular nerve cell bodies. The tela submucosae contains numerous ganglia and isolated nerve cell bodies connected by nerve fiber strands, known as the Meissner plexus (18). Finally, the tunica mucosa is innervated by the aganglionic plexus muscularis mucosae and plexus mucosus in a

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Neural invasion (NI) is a specific route of cancer spread in numerous malignancies. However, mechanisms underlying NI are unknown. The present study involving 673 patients with colon cancer is the largest one in the literature on the morphologic and functional characteristics of NI in colon cancer. On the basis of a novel NI-severity scoring system, increasing NI severity showed to be associated with poorer survival in colon cancer. However, the presence of NI in colon cancer lacks any independent prognostic significance. The novel three-dimensional-neural migration assay in the study provides functional evidence for this clinical observation by showing the lack of neuro-affinity of colon cancer cells as opposed to pancreatic cancer cells that eagerly migrate toward neurons. Therefore, based on these results, surgeons and pathologists can be less concerned with NI in the staging and treatment of colon cancer patients, whereas NI prevalence and especially NI-severity should be a major concern for patients with highly neuro-affine tumors such as pancreatic cancer.

Materials and Methods

Patient and specimen selection

673 tissue samples were collected from colon cancer patients with colonic resection in our institution between Jan. 1990 and Nov. 2005. Three groups were created as follows: group I—right colon with cancer of the coecum and ascending colon; group II—middle colon with hepatic flexure, transverse colon and splenic flexure; and group III—left colon with descending colon and sigmoid cancer (Fig. 1A). In all specimens, pathologists confirmed the diagnosis of "colon adenocarcinoma." Age of patients was between 15 and 92 years with a median age of 65 ± 11.6 years (Supplementary Table S1). Patients with additional malignancies or emergency operations were excluded. The histopathologic evaluation of tissue samples was conducted on haematoxylin and eosin-stained sections. To characterize NI, 3 different tissue specimens from the main tumor, and all lymph node sections with their respective surrounding tissue were examined. On average, 8 tissue sections were analyzed for the presence and severity of NI in each patient making the total sum of approximately 5,400 analyzed sections. Histopathologic analysis was conducted by 2 independent observers (F. Liebl and G.O. Ceyhan) blinded to patient diagnosis, followed by resolution of any differences by joint review and consultation with third observers (K. Becker and R. Langer), as reported before (6).

Reagents

McCoy’s 5a Medium, Dulbecco’s Modified Eagle’s Media (DMEM), RPMI-1640, Neurobasal medium, penicillin-streptomycin solution, trypsin-EDTA solution, B-27, and L-Glutamin from Gibco (Invitrogen); fetal calf serum (PCS; Laboratories); and ECM gel (Sigma-Aldrich).

Histopathologic evaluation—localization of NI

NI was classified according the recently established protocol for rectal cancer into the following 4 categories (22): (i) "main tumor" describes NI within the solid tumor mass (Fig. 1B-1); (ii) "peritumoral area" describes NI of nerves within the desmoplastic tissue next to the solid tumor front (Fig. 1B-2). In this category, the afflicted nerves are within the peritumoral area but lacking any direct connection to the tumor mass; (iii) It describes NI within the "mesorectal fat tissue" (Fig. 1B-3). (iv) It describes perinodal tissue surrounding metastatic or nonmetastatic "lymph nodes" (Fig. 1B-4); (v) "Auerbach’s plexus" describes cancer cell presence within the myenteric plexus (Fig. 1B-5).

Definition and establishment of a novel NI severity score in colon cancer

According to our novel established NI severity score in rectal cancer, we applied the standardized scoring system to colon cancer (22, 23). We differentiated between 3 NI stages: first, lesions in which the tumor directly touches the epineural sheet without penetrating into the perineurium: epineural tumor associations (ENA; Fig. 1C). Second, perineural invasion (PNI; Fig. 1D): defined as tumor cells found within the perineural space. Finally, the infiltration of cancer cells into the endoneurium, where they are completely encountered within the nerve fascicles: endoneural invasion (ENI; Fig. 1E). The presence of cancer cells between ganglia of Auerbach’s plexus or the direct contact of the tumor to myenteric plexus cells inside the 2 muscular...
layers was noted as: invasion to the Auerbach’s plexus (AuP; Fig. 1F).

The severity of NI was determined with the “NI severity score,” as established previously (6). For this purpose, all nerves in the entire tissue specimens were categorized and scored as noninvaded (0), ENA (1), PNI (2), or ENI (3) cancer cell invaded. NI severity score was generated by adding the number of invaded nerves (n) of the above mentioned NI severities, in the following standardized formula: Individual NI severity score = n(ENA) × 1 + n(PNI) × 2 + n(ENI) × 3 (22).

Immunohistochemistry

Consecutive 3-μm paraffin-embedded tissue sections of colon cancer and pancreatic cancer were analyzed using protein gene product 9.5/PGP9.5; 1:1,000, Dako), Artemin & NGF (1:300, Santa-Cruz), and GAP-43 (1:1,000, Millipore) antibodies via immunohistochemistry (24). In the PGP9.5 staining, 5 nerves were randomly selected, morphologically classified according to their NI severity (ENA, PNI, and ENI) and reidentified on the immunostained consecutive sections (NGF, GAP-43, and Artemin sections) and photomicrographed at ×100 magnification. Quantitative analysis of neural immunoreactivity was carried out routinely via ImageJ (NIH, USA; ref. 25).

Cell lines and cell culture

The human colon cancer cells HT29, HCT-116, SW620, and DLD-1 and the mouse rectal cancer cell CMT-93 were kindly provided by Prof. Klaus-Peter Janssen (Munich, Germany). The pancreatic cancer cell T3M4 was a kind gift by Dr. R.S. Metzgar (Durham, USA), and SU86.86 was purchased from American Type Culture Collection. The primary DRG neurons were derived from freshly isolated DRG of newborn Wistar rats (21, 26). HT29, SW620, and CMT-93 were grown in DMEM, the pancreatic cancer cells
and DLD-1 in RPMI-1640, and HCT-116 in McCoy’s 5a Medium, all supplemented with 10% FCS, 100 U/mL penicillin and 100 µg/mL streptomycin, at 37°C in a humid atmosphere, saturated with 5% CO₂.

In vitro neuroplasticity assay
Supernatant collection from cancer cells and treatment of DRG with these supernatants were conducted as shown previously (26). Briefly, serum-free supernatants were added into the neurobasal medium of ECM 24 hours after isolation and seeding at 100 µg/mL. DRG neurons were allowed to grow for further 48 hours, then fixed with 4% paraformaldehyde (Carl Roth), followed by immunofluorescence staining against the neuronal marker βIII-tubulin (anti-mouse, 1:200, Millipore) and Alexa 488 anti-mouse antibodies (Invitrogen). The entire coverslip area was photomicrographed via the Zeiss D1 Observer System. Neurite density was determined on 4 representative photomicrographs at ×200 magnification from 4 different regions of densest growth on each coverslip by overlaying a 50 × 50 µm grid and counting the fiber density per square measured in the intersecting fibers via the analySIS software (Olympus; ref. 26). The photomicrographs were taken by an investigator blinded to the treatment protocol and diagnosis, followed by analysis by a second observer (K. Kujundzic). Recombinant NGF at 10 ng/mL (Acris) was used as positive control, and serum-free neurobasal medium as negative control.

3D-migration analysis
The in vitro 3D-migration assay was conducted with colon, pancreatic cancer, and rectal cancer cells in the same technique as shown previously (21). Here, 10⁵ colon cancer cells (HT29, HCT-116, SW620, and DLD1), CMT-93 rectal cancer cells or the T3M4 and SU86.86 pancreatic cancer cells were suspended in 25 µL of an ECM gel and placed at exact 1mm distance next to DRG (3 dissociated ganglia/ECM gel). After polymerization of the gel at 37°C for 15 minutes, a 1mm-long ECM gel “bridge” was placed between the suspensions to enable interaction of the cancer cells with DRG. To exclude the possibility of an unspecific migration of the cancer cells, the cell suspensions were connected to an additional ECM gel containing no neurons (Fig. 3A). After polymerization of the ECM bridges at 37°C, neurobasal medium supplemented with 100 U/mL penicillin and 100 µg/mL streptomycin, 2% B27 and 0.5 mmol/L L-Glutamin was applied to the assay (21).

Live cell microscopy analysis of cell migration was carried out with a Zeiss observer D1 system, equipped with a CO₂ incubation chamber, an AxioCam camera, and a plan-neurolar ×10/0.3 PH1 M27 objective. For data acquisition, we investigated the activities of the colon cancer cells, the rectal cancer cell CMT-93, the pancreatic cancer cells T3M4 and SU86.86, and the DRG via digital time-lapse microscopy over a total observation time of 72 hours per movement front at ×100 magnification. Single pictures were taken at 5 minute intervals, compiled as a video and subsequently used to quantify the migratory behavior of the cancer cells.

Live cell imaging analysis. With an ImageJ-based “manual tracking” plug-in, the movements of the cancer cells were tracked. The collected data were subsequently imported to the “chemotaxis and migration tool” provided by Ibidi (www.ibidi.com). This tool uses several morphometric parameters including the velocity at which the cancer cells migrate toward neuronal structures, the euclidean distance that cancer cells cover when migrating toward the DRG and the forward-migration index (FMI) describing the targeted cancer cell migration. At the start point of each setting, 30 cells at each front were randomly selected for morphometric analysis. To not only detect single cell movements but also to express the overall tumor-cell-mass migration, the captured area occupied by the tumor cells on each image was measured. Briefly, in 10 slices of a video sequence, the area of the migration and back front was measured and plotted as percentage of the whole area. Every experimental setting with each cancer cell line was repeated 3 times.

Statistical analysis
The χ² test was used for comparisons between patient groups. The Mann–Whitney U test was conducted to compare level of semiquantitative data between 2 unrelated samples. Survival distribution was estimated and shown according to the Kaplan–Meier method. Survival differences between independent groups were statistically assessed by using the log-rank test. Cox regression analysis was used for multivariate analysis and resulting estimates of HRs were provided with 95% confidence intervals (CI). Assumption of proportional hazards was descriptively verified by investigation of partial residual plots. The relationship between GAP-43, NGF, and Artemin expression was examined using the 1-way analysis of variance. If a significant result was found, further comparisons of subgroups were conducted using Bonferroni’s multiple comparison test. Analysis of neurite density was carried out using Kruskal–Wallis followed by Dunn’s multiple comparison. Frequency of Auerbach’s plexus invasion between patients with and without NI was compared with the Mann–Whitney test. All tests were 2-sided and a P value of less than 0.05 indicated statistical significance.

Results
Prevalence of NI in colon cancer patients
NI was detected in 210 of 673 patients (31%). Sixty-eight patients had invasion only to AuP (10%), the remaining 142 patients showed varying degrees of NI (ENA, PNI ENI, and/or AuP; NI 1; 21%; Supplementary Table S1). Subgroup analysis showed a positive correlation of the invasion of AuP and NI of peripheral nerves, as the Auerbach’s plexus was additionally invaded in 39 patients (27%) of all 142 NI-1 patients, whereas only 68 of 531 patients with no invasion of peripheral nerves have shown exclusive invasion of AuP (13%, P < 0.001). The prevalence of NI did not differ among different tumor localizations in the colon (21.6% in group I, 34.2% in group II, and 36.1% in group III; Fig. 2A).
The histopathologic localization of NI in colon cancer

The highest prevalence of NI was detected within the AuP (38%) followed by the perinodal tissue (19%) and the peritumoral area (15%). Only the location “primary tumor mass” differed within the 3 groups, showing increased NI prevalence in the right colon (group I, \( P = 0.02 \)). The invasion of AuP tended to be highest in group II, but this observation did not reach statistical significance. Interestingly, NI in the perinodal tissue was almost only present when the respective lymph nodes showed metastatic cancer cell invasion.

NI severity varies among colon cancer localization

For each patient, an individual NI severity score value was calculated. In-depth analysis of the NI severity scores showed significant differences depending on the colon cancer tumor localization. NI severity scores were highest in group III (2.7 ± 9.3; mean ± SD; \( P < 0.05 \)) compared with I (0.9 ± 2.9) and II (1.3 ± 5.9, Fig. 2B).

In the present study, 114 (17%) patients had local tumor recurrence after initial R0 resection, and 131 patients (19%) revealed further local tumor progress after incomplete resection (R1/R2 resection). Tumor recurrence appeared in 74% of the patients as distant metastases, in 15% as extra or endoluminal recurrence, and in 11% as lymph node metastases and peritoneal carcinomatosis. Patients with tumor recurrence (3.9 ± 13.1) and progress (4.7 ± 9.8) revealed a noticeable significant increase in NI severity (\( P < 0.001 \)) compared with patients with no recurrence/progress (0.5 ± 2.0; Fig. 2C).
Impact of NI on prognosis and cause-specific survival

In our cohort, the median follow-up period was 64 months (inter quartile range: 26.0–101.5). The 5-year overall cause-specific survival was 69% (95% CI: 1.8%). About NI, colon cancer patients showed a significantly reduced 5-year survival probability with estimated 39% (95% CI: 4.3%) for patients with NI and 63% (95% CI: 6.0%) with only invasion of Auerbach’s plexus compared with patients without NI with estimated 78% (95% CI: 2.0%; P-value log rank test < 0.001; Fig. 2D). But, in the multivariate survival analysis including gender, age, pTNM, tumor grading/G, lymphatic vessel invasion/L, carcinoma embryonic antigen/CEA, resection status/R, and colonic localization, no impact of NI or of AuP on survival was evident when compared with patients without NI (P = 0.18 and P = 0.37 respectively; Table 1). Adjusted multivariate survival analysis did not reveal statistical significant difference between patients with NI, AuP, or patients with no neuro-cancer interactions (P = 0.40; Fig. 2E). There was also no impact of NI on survival when regarding the 3 tumor localizations (data not shown). Additional subgroup survival analysis with all 242 (36%) patients receiving adjuvant chemo- and/or radiotherapy showed a significant survival decrement for patients with NI or AuP. Five-year cause-specific survival was 56% (95% CI: 4.5%) for NI negative patients, 33% (95% CI: 5.3%) for NI positive patients and 45% (95% CI: 9.3%) for AuP patients (P = 0.001; Fig. 2F).

Colon and rectal cancer cells do not possess any major biologic affinity to neurons

To better understand the pathobiology of NI in colon cancer cells and to verify whether colon cancer cells have similar neurite targeted migration behavior as the already characterized migratory attributes of pancreatic cancer cells,
particularly the pancreatic cancer cells T3M4 (migration cell line was compared with its back front, it was obvious that migration/back front at all (Fig. 3C). The colon cancer rectal cancer cells showed no evident changes in cell velocity back front (HT29: 15.5 ± 3.5 μm, SW620: 10.37 ± 3.87 μm, and DLD-1: 0.06 ± 0.01 μm/min) when compared with the back front (HT29: 20.0 ± 5.5 μm, SW620: 20.6 ± 6.55 μm, and DLD-1: 3.0 ± 0.01 μm/min, Fig. 3C). The HCT-116 and CMT-93 cells did not show any changes within the covered distance at their migration and back front (Fig. 3D). In great contrast, pancreatic cancer cells covered considerable longer distances than colon cancer cells and rectal cancer cells covered a very prominent difference between their migration front (T3M4: 20.6 ± 6.55 μm, SW620: 38.8 ± 15.4 μm and SU86.86: 28.8 ± 4.70 μm, Fig. 3D).

Table 1. Multivariable cause specific survival analysis considering NI-categories and NI severity score

<table>
<thead>
<tr>
<th>Variable</th>
<th>Cox-proportional hazards model NI categories</th>
<th>Cox-proportional hazards model NI severity score</th>
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<td>0.8–1.9</td>
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<td>Ni severity&lt;sup&gt;+&lt;/sup&gt;</td>
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<tr>
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<sup>+</sup>Transformed to ln(NI severity score + 1).

the colon cancer cells HT29, HCT-116, SW620, and DLD-1 and the pancreatic cancer cells T3M4 and SU86.86 were investigated in the recently established 3D-ECM neural-migration assay. To detect potential differences between colon cancer and rectal cancer, additional migration analyses were conducted with the rectal cancer cell CMT-93. The different cancer cells were tracked on the migration/back front at all (Fig. 3C). The colon cancer rectal cancer cells showed a noticeable specific migration comparison to colon cancer cells HT29, HCT-116, SW620, and DLD-1 showed even higher cell velocities at their back front (HCT-116: 0.06 ± 0.003 μm/min, DLD-1: 0.02 ± 0.001 μm/min) when compared with the migration front facing DRG neurons (HCT-116: 0.05 ± 0.003 μm/min, DLD-1: 0.01 ± 0.0003 μm/min, Fig. 3C).

Cancer cell velocity. When the migration front of each cell line was compared with its back front, it was obvious that particularly the pancreatic cancer cells T3M4 (migration front/vehicle: 0.3 ± 0.06 μm/min vs. Back front/B: 0.15 ± 0.08 μm/min) and SU86.86 (M: 0.13 ± 0.03 μm/min vs. B: 0.07 ± 0.02 μm/min), and to a clearly lesser extent the colon cancer cells SW620 (M: 0.05 ± 0.01 μm/min vs. B: 0.02 ± 0.0001 μm/min) migrated much faster toward the DRG than at their back front (Fig. 3C). HT29 colon cancer cells and CMT-93 rectal cancer cells showed no evident changes in cell velocity at the migration/back front at all (Fig. 3C). The colon cancer cells HCT-116 and DLD-1 showed a higher FMI at their migration front (0.17 ± 0.01) than...
their back front (0.01 ± 0.01; Fig. 3E). The remaining colon cancer cells and the rectal cancer cell CMT-93 showed either only negative or indifferent FMI at their migration front (HCT: −0.03 ± 0.05, HT29: 0.01 ± 0.02, SW620: −0.50 ± 0.08, and CMT-93: −0.02 ± 0.02) versus back front (HCT: 0.02 ± 0.02, HT29: 0.1 ± 0.05, SW620: −0.40 ± 0.21, and CMT-93: 0.01 ± 0.03; Fig. 3E). In big contrast to colon cancer cells, both pancreatic cancer cells showed a highly neurite-targeted movement at their migration front toward the DRG neurons (T3M4: 0.25 ± 0.04 and SU86.86: 0.48 ± 0.05), which clearly surpassed the FMI values at their back front (T3M4: −0.15 ± 0.04 and SU86.86: 0.38 ± 0.06, Fig. 3E).

Captured area. As a parameter of cell mass movement, the captured area was determined. In accordance with the FMI values as indicators of specific migrations, the area occupied by all colon cancer cells (except for DLD-1) and the rectal cancer cell CMT-93 showed no noticeable increase at the back front (HCT-116: 0.42% ± 0.06%, HT29: 0.39% ± 0.86%, SW620: −3.4% ± 0.42%, and CMT-93: 0.25 ± 0.40) or the migration front (HCT-116: 0.45% ± 0.45%, HT29: 0.27% ± 0.05%, SW620: −5.4% ± 1.4%, and CMT-93: 0.88 ± 0.17; Fig. 4A and B). The DLD-1 cells showed, in accordance with its FMI, an increase in its captured area at the migration front, but this increase was a mere 0.47% ± 0.14%, which was still significantly greater than that at its back front (0.15% ± 0.36%). In great contrast to colon cancer and rectal cancer, the captured area of pancreatic cancer cells increased by 12.2% ± 2.61% (T3M4) and 8.7%
Colon cancer lacks evident amounts of neurotrophic factors

To unravel the reasons for the observed, negligible affinity of colon cancer cells to nerves as opposed to pancreatic cancer cells, an expression analysis of neurotrophic factors with chemoattractant properties, that is, NGF, the glial cell-derived neurotrophic factor family member Artemin and the neuroplasticity marker GAP-43 were quantified in nerves within human colon cancer and pancreatic cancer tissues. Nerves in pancreatic cancer showed a striking difference in the overall neural immunoreactivity with around 10-fold higher amounts of NGF (8.6% ± 2.0%), Artemin (13.6% ± 2.7%), and GAP-43 (57.8% ± 2.3%) than nerves in colon cancer tissues (NGF: 0.8% ± 0.1%, Artemin: 3.5% ± 0.7%, and GAP-43: 25.2% ± 1.2%), reflecting the relative lack of neurotrophic-chemoattractant signals in colon cancer as opposed to pancreatic cancer (Fig. 5G).

In NI, ENI in pancreatic cancer was associated with a much higher neural immunoreactivity for NGF (16.8% ± 3.8%) and Artemin (13.2% ± 2.9%), but not for GAP-43 (50.5% ± 3.4%) than in the less severe ENA (NGF: 6.5% ± 1.3%, Artemin: 0.3% ± 0.2%, and GAP-43: 48.0% ± 4.7%, Fig. 5B, D, and F). In colon cancer, only the neural immunoreactivity of NGF was significantly higher within ENI (1.9% ± 0.7%) than in ENA (0.1% ± 0.7%; Fig. 5A and B). In colon cancer, the amounts of Artemin (2.4% ± 0.9%) and GAP-43 (31.8% ± 3.7%) in nerves with ENI did not differ significantly from nerves with ENA (Artemin: 0.3% ± 0.1%, GAP-43: 23.5% ± 3.6%; Fig. 5C and E).

Pancreatic cancer and rectal cancer cells—but not colon cancer cells—induce neuroplasticity

About neuroplastic potential of pancreatic cancer and colon cancer, supernatants of the pancreatic cancer cells T3M4/SU86.86 induced a much greater DRG neurite density (4.3 ± 0.1 and 4.4 ± 0.1 neurites/2,500 µm², respectively) than colon cancer cells (SW620: 2.9 ± 0.1, DLD-1: 3.6 ± 0.1 neurites/2,500 µm²), which remained at the level of the negative control (3.6 ± 0.1 neurites/2,500 µm²; Fig. 5H). This prominent neuroplastic effect was nearly as high as the positive control neurons treated with recombinant NGF (5.2 ± 0.1 neurites/2,500 µm²). Interestingly, the rectal cancer cell CMT-93 could also induce a higher neurite density of DRG neurons (4.2 ± 0.1 neurites/2,500 µm²) than the medium/negative control, thus remaining slightly below the level of neuroplasticity induced by pancreatic cancer cells (Fig. 5H).

Discussion

The present study is the currently largest study in literature on the clinicopathologic and pathomechanistic features of NI in colon cancer. It combines a detailed histopathologic characterization of NI in colon cancer with comparative expression analysis of neural chemoattractants in colon cancer and a functional analysis of the potential neuroaffinity of colon cancer cells in comparison to pancreatic cancer and rectal cancer. It shows that the prevalence of NI correlates neither with the macroscopic nor the microscopic localization of the colon cancer. The presence of NI per se does not exert a major impact on patient prognosis, whereas increasing NI severity seems to be associated with poor survival in colon cancer. The reasons for the low NI rate in colon cancer seems to be multifold and to lie in the low neural-expression profile of neuro-chemoattractants such as NGF and Artemin and the associated low biologic affinity of colon cancer cells to invade nerves. Moreover, colon cancer cells seem to lack agents that induce neuroplastic alterations and create paths of NI in contrast to pancreatic cancer (6, 22).

Previous studies showed a prevalence of around 6% for NI in colon cancer that is indeed one of the lowest prevalence rates among all cancers, whereas in our study NI could be detected in 31.2% of all cases (16). This high discrepancy is likely to be the result of 2 key histopathologic factors: first, the presented comprehensive definition of NI and second, the fact that the main focus of our study was solely the pathobiology of NI. A major difference from other definitions of NI in colon cancer is the inclusion of invasion of Auerbach’s plexus to the possible manifestations of NI in our study. Interestingly, there is only 1 study in literature that defined perineural invasion in colon cancer as invasion only into the AuP with a prevalence of 24% (27). The invasion of peripheral nerves was neither mentioned nor examined in their study. In contrast, no other study in literature has included AuP in their definition of NI in colon cancer, which also results in lower NI prevalence rates (16, 28–30). This seems to be particularly important when one considers the association between AuP and NI in our study. Patients with invasion of peripheral nerve trunks (e.g., in the mesentery or peritumoral area) were more likely to show invasion of the AuP. Interestingly, AuP patients showed a significantly better survival than patients with NI, but still worse than patients without NI (P < 0.001). Hence, it is important to include AuP in the definition of NI to encompass all the different facets of NI in colon cancer.

In nearly all studies concerning NI and colon cancer, NI has consistently been 1 out of several histopathologic features under investigation, but not the main focus of interest (16, 29, 30). In these studies, NI was not studied by reexamination of pathology specimens but rather by data collection from original pathology reports. Indeed, targeted reexamination of pathology specimens for NI by a pathologist yields much higher prevalences of around 22% in contrast with a mere 0.5% when the data is solely derived from the presence of NI in the original pathology report (28).

But why is the prevalence of NI in colon cancer so low? One reason might be the anatomic localization of the colon. Several parts of the colon are located...
intraperitoneally, do not possess their own adjacent extrinsic plexus and, therefore, do not show high innervation density as extra-/retroperitoneal organs such as the rectum, prostate, or pancreas (31). In accordance with this anatomic relationship, NI prevalence in rectal cancer reaches up to 69%, which, in pancreatic cancer, was reported to be near 100% (6, 15, 32).

Besides enhancing the growth of neurons, neurotrophic factors are known to be potent chemoattractants (33–35). Pancreatic cancer cells are attracted by several neurotrophic factors such as NGF, glial cell-derived neurotrophic factor, and Artemin that are secreted by neuronal cells and thought to be responsible for the great neuroaffinity of pancreatic cancer cells (6, 21, 36). On the basis of our results, the...
variable neurotrophic-chemoattractive factor content of tissue-specific nerves may be one of the leading aspects in the variance of NI prevalences between different cancers. In accordance, nerves bearing severe (endoneural) invasion in both colon cancer and pancreatic cancer were characterized by higher endoneural NGF content than noninvaded nerves. Therefore, these factors, in particular NGF, seem to be crucial in understanding the differential neuroaffinity of various cancer types. In addition to the low neurotrophic factor content of colonic nerves, another major factor that contributes to low NI rates in colon cancer seems to be the overall low neuroaffinity of colon cancer cells. In the used 3D neural-migration assay, half of the studied colon cancer cells did not show any targeted migration toward DRG neurons, and the other half in no way approached the utmost fast, linear and targeted migration of the studied pancreatic cancer cells (21).

A striking observation of our study was that neither NI prevalence nor NI severity emerged as an independent prognostic factor in colon cancer. Other prognostic markers such as pN0, CEA, and resection status seem to outweigh the prognostic potential of NI. It is possible that the effect of NI on survival in the univariate analysis can be explained by the additional influence of a high percentage of advanced tumors within the NI-positive collective. This is supported by the correlation of high NI severity scores with recurrence or progress, as tumor residuals are potent predictors of poor survival. In contrast to our results, other studies previously confirmed NI as a prognostic factor in colon cancer (28). However, most of these studies have quite low sample size, limited to certain tumor stages and do not distinguish between rectal and colon cancer, which represent biologically different cancer entities (27–30).

In this context, the present study particularly underlines the importance of NI severity for patient prognosis in colon cancer, because obviously, the severity of NI allows more accurate estimates of patient prognosis than NI prevalence alone. A further advantage of the NI severity score shows itself in the detection of differences between various colon tumor localizations: We detected an almost equal and homogeneous distribution of NI in the 3 groups, with only a tendency to increased NI prevalence within group III. However, the NI severity score in group III was remarkably higher than in group I. This finding is supported by the fact that left colon cancer seems to present a greater genetic and chromosomal instability, higher p53 mutation rate and thus higher aggressiveness than colon cancer of the right side (37, 38). Similarly, also in rectal cancer, we were able to confirm a significant prognostic potential of NI and a higher incidence of local recurrence in conjunction with escalating NI severity scores (22). To really elucidate a potential difference between the neuro-affinity of rectal cancer and colon cancer, the 3D migrations analyses were extended to include rectal cancer cells (CMT-93). Here, Rectal cancer cells did not differ from colon cancer cells in terms of their neuro-affinity, suggesting that they also seem to fail to respond to mediators originating from neural structures. On the other hand, rectal cancer cells were able to stimulate increased neurite density of DRG in contrast to colon cancer cells. Therefore, the reason for higher overall severity of NI in rectal cancer versus colon cancer and its significant impact on patient survival may actually be because of the capability of rectal cancer to induce neuroplasticity and thus higher rectal innervation density with higher natural proximity of large-caliber nerve trunks in the mesorectum than the nerves of the mesocolon.

Altogether, by using a consistent definition for NI, we could verify the low prevalence of NI in colon cancer and show the lack of any natural affinity between colon cancer cells and neuronal structures as a possible explanation for its low prevalence. Finally, not the prevalence, but rather the severity of NI proved to be a better predictor of survival in colon cancer. Therefore, surgeons and pathologists should pay more attention to the severity of NI and not necessarily to its sole presence in the staging and treatment of colon cancer patients.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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References
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The Severity of Neural Invasion Is Associated with Shortened Survival in Colon Cancer

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