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Inactivation of axon guidance molecule netrin-1 in human colorectal cancer by an epigenetic mechanism

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ABSTRACT

Netrin-1, the protein product of the *NTN1* gene, is an axon guidance molecule implicated in regulation of cell survival and tumorigenesis. Expression of the netrin-1 receptors deleted in colorectal cancer (*DCC*) and uncoordinated 5 homolog (*UNC5H*) is frequently silenced in colorectal cancer (CRC) by either loss of heterozygosity or epigenetic mechanisms. However, netrin-1 expression and regulation in CRC are mostly unknown. Here, we report that *NTN1* expression is significantly reduced in most CRC tissues compared to the adjacent normal intestinal mucosa, and that *NTN1* DNA methylation is significantly higher in CRCs (24.6%) than in the adjacent normal intestinal mucosa (4.0%). In 6 CRC cell lines, *NTN1* expression is low. Treatment with 5-Aza-2′-deoxycytidine increased expression of *NTN1* in CRC cell lines, indicating that DNA methylation represses *NTN1* transcription in CRCs. *NTN1* DNA hypermethylation was significantly associated with advanced CRC disease. Median netrin-1 serum levels were significantly decreased in CRC patients (330.1 pg/mL) compared with normal individuals (438.6 pg/mL). Our results suggest that netrin-1 is a candidate biomarker for CRC.

1. Introduction

Colorectal cancer (CRC) is the second most deadly and third most commonly diagnosed cancer in the world, according to GLOBOCAN 2020 [1]. Currently, early diagnosis of CRC is achieved by screening colonoscopy, which is extremely effective, but invasive. It would be useful to detect CRC with a non-invasive procedure. Like other epithelial cancers, CRC is believed to arise through the successive accumulation of genetic and epigenetic alterations in oncogenes, tumor suppressor genes, and DNA repair pathway genes [2,3]. DNA hypermethylation of promoter-associated CpG islands is a frequent and early mechanism

Abbreviations: DCC, deleted in colorectal cancer; UNC5H, uncoordinated 5 homolog; CRC, colorectal cancer; DSCAM, down syndrome cell adhesion molecule; VEGF, vascular endothelial growth factor; EC, epithelial cell; DAC, 5-Aza-2′-deoxycytidine; APC, adenomatous polyposis coli

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of gene inactivation in CRC and other cancers, but no DNA methylation marker is a clinically applicable biomarker of CRC [4,5].

Netrins are laminin-like proteins, first identified as axonal guidance molecules in *Caenorhabditis elegans* [6]. The netrin family consists of four secreted molecules, netrin-1, netrin-3, netrin-4, and netrin-5, and two membrane-anchored members, netrin-G1 and netrin-G2. Netrin-1, secreted from floor plate during brain development, establishes a gradient that attracts to the midline the commissural axons that express netrin receptors [6,7]. Several receptors mediate the function of netrin family proteins, including uncoordinated-5-Homologs (UNC5H1-4/A-D), deleted in colorectal cancer (DCC), the DCC orthologue neogenin (NEO1), and Down syndrome cell adhesion molecule (DSCAM). These receptors are "dependence receptors" as they promote cell proliferation, migration, and survival when activated by the ligand, but trigger apoptosis when the ligand is limiting [8]. In the gut, netrin-1 is predominantly expressed at the bottom of the intestinal crypt, whereas DCC is expressed all along the villus. Within the crypt, netrin-1 binding to DCC induces crypt cell survival. At the villus tips, where netrin-1 levels are low, DCC promotes apoptotic cell death [3,9]. *DCC* was identified as a candidate tumor suppressor in CRC with a deletion in chromosome 18q21 [10,11], and loss of netrin-1 receptor expression was associated with a poor prognosis in patients with CRC [3,9]. Also, expression of *UNC5H* genes is silenced in CRC, either by loss of heterozygosity or epigenetic mechanisms [12].

Netrin-1 mRNA and protein levels are increased in metastatic breast cancer, non-small cell lung cancer, and pancreatic adenocarcinoma, where netrin-1 functions as an oncogene and may be associated with loss of netrin-1 receptors [13–16]. Recently, we have shown that netrin-1 promotes invasiveness and angiogenesis in glioblastoma and medulloblastoma by activating the cysteine protease cathepsin B [17–19]. However, the function of netrin-1 in CRC is not clear. In this report, we demonstrate that netrin-1 mRNA and protein levels are significantly reduced in most CRC tissues compared to the adjacent normal intestinal mucosa, and that *NTN1* methylation is significantly greater in CRC tissues compared to the adjacent normal mucosa. The demethylating drug 5-Aza-2′-deoxycytidine restores *NTN1* expression in CRC cell lines, suggesting that *NTN1* expression is silenced by DNA methylation in CRC. We also find that serum levels of netrin-1 are significantly decreased in patients with CRC compared with normal individuals and are predictors of disease severity. Together, our results suggest that netrin-1 reduction is a candidate biomarker of CRC.

2. Materials and methods

2.1. CRC samples

Tissue samples of CRC and matched normal intestinal mucosa were collected during a colonoscopy from 25 patients at Nippon Kokan Fukuyama Hospital (Fukuyama, Japan), prior to initiation of cancer therapy, and were stored at −80 °C until analyzed. Peripheral blood samples, collected from the CRC patients in endotoxin-free silicone-coated tubes (Sekisui Medical Co., Tokyo, Japan) a few days prior to surgery, were allowed to clot at room temperature for 30 min before centrifugation (3000 rpm, 10 min); serum was removed and stored at −80 °C until analyzed. To establish a normal reference range, serum netrin-1 concentrations were measured in 30 healthy volunteer blood donors. This study was approved by the Institutional Review Board of Hiroshima International University (approved IRB protocol number: 18-015) and Nippon Kokan Fukuyama Hospital (approved IRB protocol number: 2018-01).

2.2. NTN1 expression analysis with TCGA datasets

Paired RNA-seq datasets of rectum adenocarcinoma (TCGA-READ)/colon adenocarcinoma (TCGA-COAD) and adjacent normal tissue were downloaded from The Cancer Genome Atlas (TCGA) using the data transfer software, GDC-client (version 1.5.0). Paired samples of solid tumor and normal tissue from 9 patients of TCGA-READ and 46 patients of TCGA-COAD are listed in Supplementary Table 4. Datasets were unzipped using the Linux command "*gzip -cd*" on a high-performance computer, NIH Biowulf. *NTN1* expression levels (Fragments Per Kilobase pair per Million reads, FPKM) were extracted from each dataset using the Linux command "*grep -w NTN1*". Extracted data files were combined by the Linux command "*cat*". The results were visualized using Microsoft EXCEL.

2.3. DNA methylation analyses with TCGA datasets

Datasets of DNA methylation arrays (Illumina Human Methylation array 450 k or 27 k) from paired rectum/colon solid tumor and paired normal tissue were downloaded from TCGA-READ and TCGA-COAD using the GDC-client through NIH Biowulf. Individual datasets are listed in Supplementary Table 5. Datasets were unzipped using "*gzip -cd*", and DNA methylation status in a CpG island near the promoter of *NTN1* (probe cg19564877) was extracted using the Linux command "*grep -w cg19564877*". The extracted data files were combined using the Linux command "*cat*". The results were visualized using Microsoft EXCEL.

For correlation analysis between *NTN1* DNA methylation and TNM stage (Fig. 2D), datasets of DNA methylation arrays from normal tissues of 51 patients and tumors of 617 patients were downloaded from TCGA-READ and TCGA-COAD (see Supplementary Table 6). Extracted data files from each patient were combined as a CSV file on NIH Biowulf. The combined CSV file was opened by Microsoft EXCEL, and data were summarized based on TNM stage.

2.4. Correlation analysis of DNA methylation and mRNA expression of NTN1

Datasets of RNA-seq and DNA methylation arrays from the same patients were downloaded from TCGA-READ and TCGA-COAD using the GDC-client. The used datasets are listed in Supplementary Table 7. Datasets were unzipped using the Linux command "*gzip -cd*". *NTN1* gene expression and the "sample_submitter_id" were extracted from RNA-seq datasets as described in the previous method section. DNA methylation levels and the "sample_submitter_id" were extracted from datasets of DNA methylation arrays described in the previous method section. Extracted data files of *NTN1* gene expression and DNA methylation of *NTN1* CpG island proximal to the promoter were combined into a CSV file using the Linux command "*cat*". A table is generated, which has 3 columns; 1st column is "sample_submitter_id", 2nd column is *NTN1* FPKM values, and 3rd column is DNA methylation level of *NTN1* CpG island proximal to the promoter. The data were visualized as a dot plot shown in Fig. 2C.

2.5. Bisulfite modification and sequence of NTN1 promoter region

Bisulfite modification of genomic DNA from cultured cells was performed by EpiTect Bisulfite Kits (Qiagen, Chatsworth, CA). We first identified tentative CpG sites in the *NTN1* promoter region (chr17: 9,020,851–9,021,600). There were 68 CpG sites within the promoter region (nucleotides −1523 to −774) analyzed in this study. The nucleotide number +1 is defined as the start codon of *NTN1*. Bisulfite-modified DNA was amplified using the primer set listed in Supplementary Table 1. Each of the PCR products was subsequently cloned using the pT7Blue T-vector (Sigma-Aldrich). To quantify the percentage of methylated CpG sites, 8 clones were sequenced and identified the bisulfite converted DNA.

2.6. Statistical analysis

GraphPad Prism 6 statistical software (GraphPad Software; San Diego, CA) was used for statistical analyses. The results are represented as medians and interquartile ranges were used to summarize the tumor patient data, and controls were compared using the nonparametric Mann-Whitney *U* test. Diagnostic accuracy was assessed with receiver operating characteristic (ROC) curve analysis, and the Youden Index was used to identify cut-off values. $P < 0.05$ was considered statistically significant.

3. Results and discussion

We first evaluated netrin-1 expression in CRC. Levels of *NTN1* mRNA were derived from TCGA data sets of paired samples of CRC tissues and normal adjacent intestinal mucosa (n = 55). *NTN1* is expressed at significantly ($P = 3.67 \times 10^{-13}$) lower levels in CRC tissue samples compared to the adjacent normal intestinal mucosa (Fig. 1A and B). We confirmed this observation in additional 25 paired samples of CRC and normal adjacent intestinal mucosa ($P < 0.05$, Fig. 1C), by using qRT-PCR (Supplementary Tables 1 and 2).

Using these 25 paired samples of CRC and adjacent normal intestinal mucosa, we examined expression of netrin-1 receptors. Netrin-1 has multiple receptors [6]. By qRT-PCR, netrin-1 receptors *DCC*, *NEO1*, *UNC5B, UNC5C* and *UNC5D* were expressed in the normal mucosa, whereas *DSCAM* and *UNC5A* were not expressed. *NEO1* and *UNC5B* were expressed at the highest levels in the normal mucosa. Expression of all 5 receptors was significantly decreased in CRCs compared with the normal mucosa (Fig. 1C). Angiogenesis contributes to tumor progression and metastasis. Vascular endothelial growth factor (VEGF) is a potent angiogenic factor. Circulating VEGF-A levels are abnormally increased in patients with CRC and anti-VEGF therapy is effective at reducing tumor growth in patients with CRC [20–22]. Unlike netrin-1 and its receptors, expression of *VEGFA* was elevated in CRC samples compared to the normal adjacent intestinal mucosa ($P < 0.05$, Fig. 1D), consistent with previous reports [23,24]. Western blot analysis in 3 randomly selected CRC samples showed that protein levels of netrin-1 and UNC5C are reduced in CRCs compared with the corresponding normal colonic mucosa (Fig. 1E). Together, these results show that netrin-1 mRNA and protein levels are reduced in CRC tissues compared to the normal adjacent mucosa.

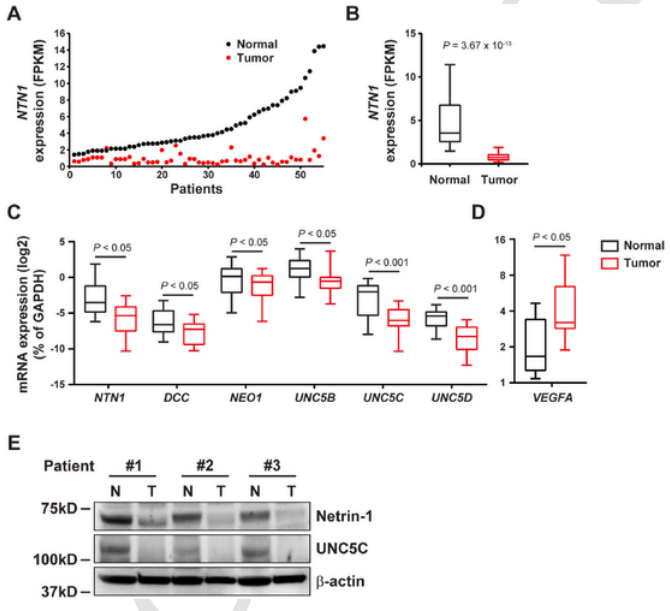

Fig. 1. *NTN1* expression is reduced in CRC compared to normal colon. A,B, *NTN1* expression in paired samples of CRC and normal colonic tissues by RNA-seq from TCGA database (n = 55). Results for individual CRC and paired normal colon tissue samples are displayed as dots at the same position in the x axis (A). Mean mRNA levels in CRC and paired normal colonic epithelium tissues are displayed as box-and-whisker plots (B). The horizontal line indicated the median (B). C,D, Relative mRNA levels of *NTN1* and its receptors and *VEGFA* measured by qRT-PCR in paired samples of CRC and normal colon tissues (n = 25). mRNA levels were normalized to *GAPDH* mRNA. E, Netrin-1 and UNC5C protein levels in three paired samples of CRC and normal colon tissues (normal: N, tumor: T) were analyzed by Western blot analysis.

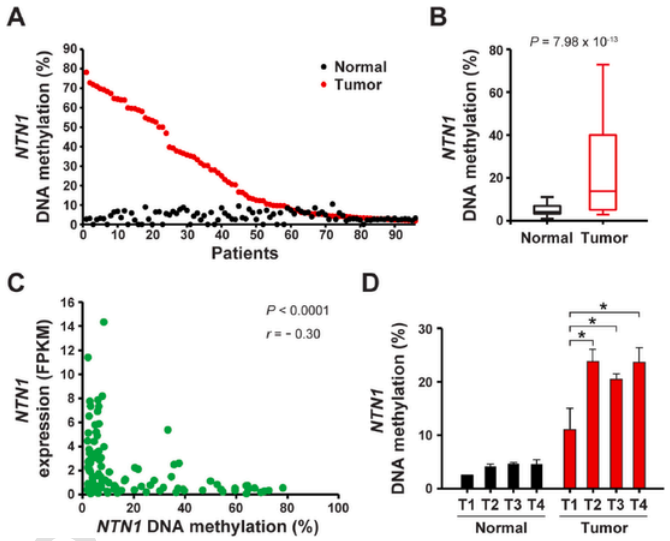

Fig. 2. *NTN1* DNA is frequently hypermethylated in CRCs. A,B, DNA methylation levels in *NTN1* CpG island (chr17: 8,865,316–8,866,185) located proximal to the *NTN1* promoter were analyzed from the Human Methylation 450K Array (HM450, Illumina) in TCGA datasets of paired samples of CRC and normal colon tissues (n = 96). Results from individual pairs are shown as dots (A) and cumulative group results are shown as box-and-whisker plots (B). C, Correlation between *NTN1* gene expression and DNA methylation in colorectal cancer (n = 95) and adjacent normal colon tissues (n = 39). D, Correlation between *NTN1* DNA methylation and TNM cancer stage. Datasets were from TCGA database (see Supplementary Tables 5–7). Data represent the mean ± SD [normal: T1 (n = 1), T2 (n = 10), T3 (n = 69), T4 (n = 7); tumor: T1 (n = 20), T2 (n = 108), T3 (n = 430), T4 (n = 72)], $^*P < 0.05$.

Methylation-induced gene silencing frequently occurs in cancer [12,25,26]. We examined the status of *NTN1* methylation in CRC samples and paired samples from the normal adjacent intestinal mucosa (n = 92) using available TCGA datasets derived from the Human Methylation 450K or 27K Array (Illumina). This comparison, focused on the level of DNA methylation within the CpG islands at the 5′ untranslated region of *NTN1* (position −692 to +2061 relative to the transcription start site), revealed that the region was hypermethylated in tumor samples compared with the paired normal intestinal samples (mean β values: 24.7% in tumors and 4.01% in normal tissue, $P = 7.98 \times 10^{-13}$, Fig. 2A and B). The mean percentage of CpG methylation of *NTN1* was inversely correlated with *NTN1* expression level (Pearson's $r = -0.30$), suggesting that DNA methylation represses *NTN1* transcription in CRCs (Fig. 2C). In addition, hypermethylation of *NTN1* was frequently observed in patients with TNM stages II-IV (Fig. 2D), indicating that DNA hypermethylation of *NTN1* was significantly associated with advanced CRC.

By qRT-PCR, we examined *NTN1* expression in normal colonic epithelial cells (ECs) and 6 CRC cell lines. Consistent with the result of CRC patient samples (Fig. 1), all CRC cell lines showed significantly ($P < 0.01$) reduced *NTN1* levels compared to normal colon ECs (Fig. 3A). Since methylation-induced gene silencing is frequently observed in human cancers, we examined the DNA methylation status in normal

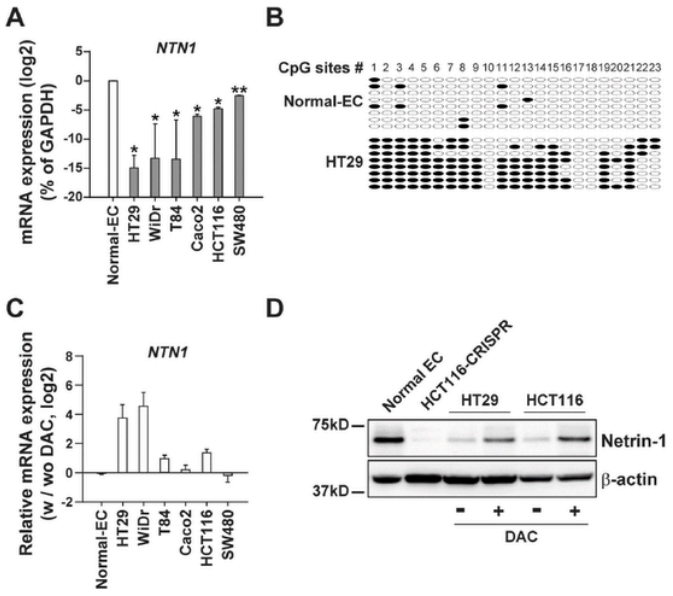

Fig. 3. Effects of DNA hypermethylation on *NTN1* expression in CRC cell lines. A, *NTN1* mRNA levels were measured in normal colon EC and CRC cell lines. mRNA levels were normalized to *GAPDH* mRNA. *$P < 0.001$, **$P < 0.01$. B, Bisulfite sequencing of normal EC and HT29 cells. Empty circles indicate unmethylated CpG sites (#1–23), filled circles represent methylated CpG sites (#1–23). C, Cells incubated with (w) or without (wo) DAC (10 μM) for 3 days were analyzed by qRT-PCR (fold change, w/wo DAC). *NTN1* mRNA levels were normalized to *GAPDH* mRNA. D, CRC cell lines (HT29 and HCT116) were treated with DAC (10 μM) for 3 days and cell lysates were analyzed by Western blotting. Normal EC and HCT116 cells after CRISPR/Cas9-mediated netrin-1 knockout are used as controls for Western blotting.

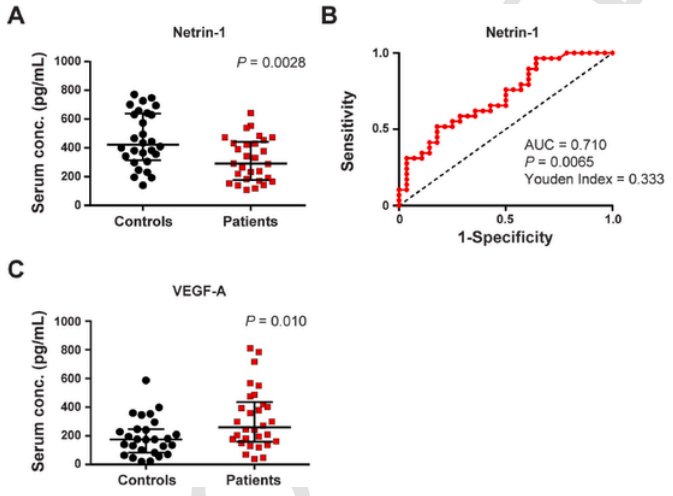

Fig. 4. Serum netrin-1 levels are reduced in patients with CRCs compared to blood donors. Serum netrin-1 (A) and VEGF-A (C) levels were quantified by ELISA in patients with CRCs (n = 31) and normal individuals (n = 30). B, Specificity of and sensitivity of netrin-1 serum levels at predicting CRC. ROC curve analysis for determining the predictive value of serum netrin-1 concentration for CRC diagnosis.

colon ECs and HT29 cells. By bisulfite sequencing, the *NTN1* promoter region (nucleotides −1523 to −1250) was hypermethylated in 64.1% (15 of 23) CpG sites, compared to 5.4% (1 of 23) CpG sites in normal colon ECs (Fig. 3B and Supplementary Fig. 1). We cultured CRC cell lines and normal colon ECs with decitabine (5-Aza-2′-deoxycytidine, DAC). Treatment with DAC resulted in a significant increase of *NTN1* in 5 CRC cell lines. However, DAC had a minimal effect on *NTN1* expression in normal colonic ECs and in SW480 cells (Fig. 3C). Western blot analysis showed that DAC treatment increased netrin-1 protein levels in the HT29 and HCT116 cell lines (Fig. 3D).

Netrin-1 receptors were also measured (Supplementary Fig. 2A). Five netrin-1 receptors were detected in CRC cell lines, whereas *UNC5D* and *DSCAM* were not detected. *DCC* and *UNC5A-C* were decreased in CRC cell lines compared to the normal colonic ECs (Supplementary Fig. 2A). Previous studies reported that netrin-1 receptors *DCC* and *UNC5A-C* are down-regulated in multiple cancers, including CRC, and suggested that loss of netrin-1 receptors is caused by loss of heterozygosity or epigenetic processes [12,27]. Consistent with these previous results, DAC treatment restored mRNA expression in *DCC* and *UNC5A-C* receptors in the CRC cell lines (Supplementary Fig. 2B). Altogether, these results suggest that DNA hypermethylation regulates the transcriptional repression of netrin-1 ligand and receptors in human CRC.

At present, it is not clear whether netrin-1 expression plays a role in colorectal cancer development [28,29]. Several lines of evidence supported a crucial role of netrin-1 during colorectal tumorigenesis by regulating apoptosis [28]. Overexpression in the mouse gut of netrin-1, under the control of the gut-specific liver fatty acid-binding protein gene (Fabpl) promoter, inhibited intestinal epithelial cell death and promoted formation of hyperplastic and neoplastic lesions [28]. Additionally, inhibition of netrin-1-mediated apoptosis was associated with tumor progression in mice carrying the adenomatous polyposis coli (APC) mutation [28]. Inflammatory bowel disease in a mouse model was associated with increased netrin-1 expression in the colonic epithelial cells via the NF-kB pathway and with colorectal cancer development [29]. These apparently inconsistent results might be attributable to forced expression of netrin-1 in the mouse model, and on netrin-1 affecting various cell types, including endothelial cells and immune cells, all contributing to tumor development [6,7]. Further work is needed to dissect the mechanisms underlying the pro-tumorigenic effects of ectopic netrin-1 expression mice. Another potential explanation for these discrepant results is that reduction of netrin-1 expression may inhibit the survival of highly proliferative cells at the base of the intestinal crypt because netrin-1/DCC interactions are essential for tight regulation of epithelial cells proliferation and differentiation in the intestine. Thus, we can speculate that the loss of the proximal-to-distal netrin-1 gradient in a netrin-1-deprived environment impairs intestinal cellular homeostasis, eventually initiating malignant transformation.

Recent studies show that serum netrin-1 levels may be clinically useful as a biomarker for human cancers [30]. We measured serum levels of netrin-1 by ELISA in patients with CRC and healthy blood donors. The baseline demographic, clinical, and CRC characteristics of the participants are presented in Supplementary Table 2. Accuracy of the netrin-1 ELISA was determined by spiking and recovery testing (Supplementary Table 3). The median serum netrin-1 concentration was 438.6 pg/mL (IQR, 332.1–666.1) for control and 330.1 pg/mL (IQR, 181.9–472.3) for patients with CRC ($P = 0.0028$), indicating that serum netrin-1 levels are significantly decreased in CRCs (Fig. 4A). A receiver operating characteristic (ROC) curve analysis indicated that serum netrin-1 provided moderate diagnostic accuracy in differentiating tumor from control groups [area under the curve (AUC): 0.710, $P = 0.0065$]. The Youden Index revealed that the optimal cut-off value is < 293.8 pg/mL (Fig. 4B). We also measured serum VEGF-A levels which served as an experimental positive control [24]. Serum VEGF-A levels were elevated in patients with CRC (median 271.5 pg/mL, IQR, 161.5–474.3) compared to controls (median 178.1 pg/mL, IQR, 97.3–347.4, $P = 0.010$) (Fig. 4C).

In summary, we show here that CRC patients samples exhibit reduced expression of *NTN1* attributable to DNA methylation. Our data further indicate that detection of *NTN1* DNA hypermethylation and serum levels of netrin-1 may provide biomarkers for predicting CRC progression. Further studies are needed to define the function of netrin-1 inactivation on tumor cell proliferation, invasiveness, and angiogene-

sis in CRC. However, these data provide a basis for investigating netrin-1 as a potential new target for treatment of CRC.

Declaration of competing interest

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2022.04.069.

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