Genetic and epigenetic alterations of netrin-1 receptors in gastric cancer with chromosomal instability

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Abstract

Background: The gene expressions of netrin-1 dependence receptors, DCC and UNC5C, are frequently downregulated in many cancers. We hypothesized that downregulation of DCC and UNC5C has an important growth regulatory function in gastric tumorigenesis.

Results: In the present study, a series of genetic and epigenetic analyses for DCC and UNC5C were performed in a Japanese cohort of 98 sporadic gastric cancers and corresponding normal gastric mucosa specimens. Loss of heterozygosity (LOH) analyses and microsatellite instability (MSI) analysis was applied to determine chromosomal instability (CIN) and MSI phenotypes, respectively. More than 5 % methylation in the DCC and UNC5C promoters were found in 45 % (44/98) and 32 % (31/98) gastric cancers, respectively, and in 9 % (9/105) and 5 % (5/105) normal gastric mucosa, respectively. Overall, 70 % (58 of 83 informative cases) and 51 % (40 of 79 informative cases) of gastric cancers harbored either LOH or aberrant methylation in the DCC and UNC5C genes, respectively. In total, 77 % (51 of 66 informative cases) of gastric cancers showed cumulative defects in these two dependence receptors and were significantly associated with chromosomal instability. Both DCC and UNC5C were inactivated in 97 % of CIN-positive gastric cancers and in 55 % of CIN-negative gastric cancers.

Conclusions: Defect in netrin receptors is a common feature in gastric cancers. DCC alterations are apparent in the early stages, and UNC5C alterations escalate with the progression of the disease, suggesting that the cumulative alterations of netrin-1 receptors was a late event in gastric cancer progression and emphasizing the importance of this growth regulatory pathway in gastric carcinogenesis.

Keywords: Gastric cancer, Methylation, Chromosomal instability, DCC, UNC5C, Netrin-1 receptors

Background

Global estimates of cancer incidence rank gastric cancer as the fourth most common malignancy and the second most common cause of cancer-related deaths worldwide [1]. Gastric cancer is a heterogeneous disease with multiple environmental etiologies and with alternative pathways of carcinogenesis [2, 3]. One of the major etiological risk factors for gastric cancer is Helicobacter pylori (H. pylori) infection. Previous reports indicated a 93.1–100 % infection rate for H. pylori in patients with gastric cancer, while only 1.2–2.8 % of individuals infected with H. pylori develop gastric cancer [4–7].

Current knowledge on the molecular mechanisms underlying gastric carcinogenesis indicates one major epigenetic instability pathway and two major genetic instability pathways [8]. The major epigenetic instability pathway is defined as the CpG island methylator phenotype (CIMP), which was initially described in colorectal cancer and also observed in a subset of gastric cancers and which harbors a critical degree of aberrant promoter hypermethylation associated with transcriptional silencing of multiple tumor suppressor genes [9, 10].
two major genetic instability pathways include microsatellite instability (MSI) and chromosomal instability (CIN) [8]. MSI is defined as the presence of replication errors in simple repetitive microsatellite sequences caused by mismatch repair (MMR) deficiencies. One is Lynch syndrome caused by germline mutations in MMR genes and another is sporadic MSI caused mainly by promoter hypermethylation in the MLH1 gene [10, 11]. On the other hand, CIN, which is characterized by chromosomal alterations—either qualitative or quantitative—is a more common pathway that may comprise clinicopathologically and molecularly heterogeneous tumors [8].

The Cancer Genome Atlas Research Network recently divided gastric cancers into four subtypes [12]. Tumors were first categorized by Epstein–Barr virus (EBV)-positivity (9 %), then by MSI-high status, hereafter called MSI-positive (22 %), and the remaining tumors were classified by degree of aneuploidy into those termed genomics strictly stable (20 %) or those exhibiting CIN (50 %). EBV-positive cancers as well as MSI-positive cancers were known to cluster each on its own, exhibiting extreme CIMP. Differences between the EBV-CIMP and MSI-associated gastric-CIMP methylation profiles are exemplified by the fact that all EBV-positive tumors assayed displayed CDKN2A (p16INK4A) promoter hypermethylation but lacked the MLH1 hypermethylation characteristic of MSI-associated CIMP.

With respect to CIN characterized by copy number changes in chromosomes, Deng et al. used high resolution genomic analysis to profile somatic copy number alterations in a panel of 233 gastric cancers (primary tumors and cell lines) and 98 matched gastric non-malignant tissues. Regarding broad chromosomal regions, the most frequently amplified region included chromosomes 1q, 5p, 6p, 7p, 7q, 8q, 13q, 19p, 20p, and 20q, and the most frequently deleted region included chromosomes 3p, 4p, 4q, 5q, 6q, 9p, 14q, 18q, and 21q [13].

Frequently deleted chromosomal regions are usually characterized by loss of heterozygosity (LOH) and suggest the presence of tumor suppressor genes [14, 15]. LOH on chromosome 18q21 is found in 30–71 % of gastric cancers [13, 16–18], and DPC4 (Smad4)/DCC have been postulated to be the major targets. DPC4 (Smad4), a tumor suppressor gene, exhibits frequent mutations accompanied by LOH in approximately 20 % of pancreatic cancers [19], but no mutations have been reported in gastric cancers [20]. In contrast, few studies have focused on DCC gene alterations, and its genetic/epigenetic status still remains virtually unexplored in gastric cancer, partly because of the length and complexity of this gene [21]. Interestingly, recent studies have demonstrated that DCC as well as UNC5C serve as dependence receptors for netrin-1, thus, reinforcing their potential role as tumor-suppressors in human cancers [22–25].

DCC receptors are distributed along the length of the epithelial in the intestine, whereas netrin-1 is differentially expressed, forming a gradient within the gastrointestinal tract [24]. A high concentration of netrin-1 is present at the crypt base where stem cells and transient amplifying cells reside. By contrast, a low concentration of netrin-1 exists at the tip of the villi, where many cells are undergoing apoptosis and sloughing-off. This netrin-1 gradient was examined further using transgenic mice to determine if netrin-1 is responsible for regulating DCC-induced apoptosis in the intestinal epithelium [24]. The study by Mazelin et al. indicated that netrin-1 overexpression caused a decrease in intestinal epithelial cell death, whereas no increase in proliferation and differentiation of cells was observed. By contrast, netrin-1–mutant newborn mice exhibited increased cell death. Taken together, these data support the concept that netrin-1 regulates apoptosis through the DCC-dependence receptor in the intestine. However, netrin-1 is unlikely to be a direct regulator of intestinal homeostasis, given that normal epithelial organization is not disrupted by netrin-1 overexpression [24].

Similar to DCC receptors, other netrin-1 receptors, including UNC5A, UNC5B, and UNC5C, were also discovered as putative tumor suppressor genes in various tumors, including gastric cancer [26, 27]. In particular, a twofold downregulation of UNC5C expression compared with the corresponding normal tissues was observed in approximately 70 % of gastric cancer cases [26]. This region is located at 4q21–23, which is often a site of deletion in gastric cancer and is associated with epigenetic gene inactivation, such as promoter methylation [26–28].

In this study, we hypothesized that downregulation of DCC and UNC5C plays an important growth regulatory function in gastric tumorigenesis, which we addressed by investigating a panel of gastric cancer cell lines and clinical specimens from patients with gastric cancer. Herein, we report that the majority of gastric cancers show loss of both netrin-1 receptors. We also provide data suggesting that the inactivation of these receptors is mediated through both genetic and epigenetic mechanisms. Cumulative defects in these two dependence receptors are significantly associated with the CIN phenotype, emphasizing the importance of these novel findings and this growth regulatory pathway in gastric carcinogenesis.

Results
Characteristics of gastric cancer patients
Of 98 gastric cancer patients, 34 patients were female (35 %), and 48 tumors were pathologically diagnosed as differentiated (49 %) (Table 1). With regard to TNM stage, 18, 29, 37, and 14 gastric cancer patients were classified as stage I, II, III, and IV, respectively. By tumor
genetic analyses, 13 gastric cancers were categorized as displaying microsatellite instability (MSI; 13 %). The mean LOH ratio of the 98 tumors was 0.24 (standard deviation (SD), ±0.3).

CIN phenotype was categorized by calculating the LOH ratio of the informative markers of the seven polymorphic microsatellite sequences, independently from the 4q and 18q loci. When a tumor showed a LOH ratio higher than 0, the tumor was categorized as CIN-positive. By this criterion, 50/98 tumors (51 %) were classified as CIN-positive.

Direct sequencing of gastric cancer specimens revealed the proportion of KRAS, BRAF, and PIK3CA mutations (Table 1). Mutations were detected in the KRAS codon 12 (5 %, N = 5/98) and codon 13 (1 %, N = 1/98); BRAF codon 600 (0 %, N = 0/98); PIK3CA codon 545 (1 %, N = 1/98); and codon 1047 (3 %, N = 3/98). KRAS codon 12 mutations consisted of G12D (35G to A, N = 4) and G12R (34G to C, N = 1), and codon 13 mutations included G13D (38G to A, N = 1). Interestingly, one tumor displayed both KRAS codon 12 and 13 mutations (Additional file 1: Figure S1A).

PIK3CA exon 9 mutations comprised E545K (1633G to A, N = 1), while exon 20 mutations comprised H1047R (3140A to G, N = 3). Furthermore, we determined the infection status of H. pylori by recovering the cagA genotype (Additional file 1: Figure S1B). Through this analysis, we could recover the cagA sequence from 70 gastric cancer tissues (71 %).

**Table 1 Characteristics of gastric cancer patients**

| Characteristic | Percentage (No.) |
|---------------|------------------|
| Age           | Mean age (SD) 65.1 (11.8) |
| Gender        | Female 35 (34) Male 65 (64) |
| Histology     | Diff 49 (48) Undiff 51 (50) |
| Stage         | IA/IB 18 (18) IIA/IIIB 30 (29) IIIA/IIIB/IIIC 38 (37) IV 14 (14) |
| T             | T1a/1b 14 (14) T2 14 (14) T3 28 (27) T4a/4b 44 (43) |
| N             | N0 27 (26) N1 35 (34) N2 24 (24) N3 14 (14) |
| Distant metastasis | Negative 86 (84) Positive 14 (14) |
| MSI           | MSI 13 (13) Non-MSI 87 (85) |
| LOH Ratio     | Mean Ratio (SD) 0.24 (0.3) |
| CIN           | Positive 51 (50) Negative 47 (46) Not informative 2 (2) |
| KRAS          | Mutant 5 (5) Wild 95 (93) |
| BRAF          | Mutant 0 (0) Wild 100 (98) |
| PIK3CA        | Mutant 4 (3) Wild 96 (94) |
| H.pylori      | Positive 71 (70) Negative 29 (28) |

Methylation status of DCC in gastric cancer specimens and association with clinicopathological features

We investigated DCC methylation status in 98 gastric cancers and 105 normal gastric mucosa specimens. Location of the DCC gene and the results of a panel of representative combined bisulfite restriction analyses (COBRA) are depicted in Fig. 1a–b; these results were analyzed as continuous variables (Fig. 1c). We found that 56/98 gastric cancers (57 %) and 31/105 normal gastric mucosa specimens (29.5 %) displayed more than 1.0 % methylation in the DCC promoter. The mean methylation level was 18.3 % [95 % confidence interval (CI), 14.5–22.2 %] among gastric cancer tissues that displayed over 1.0 % methylation in the DCC promoter and 4.9 % (95 % CI, 3.3–6.5 %) in the corresponding normal gastric mucosa specimens that displayed over 1.0 % methylation (P < 0.0001, Wilcoxon/Kruskal–Wallis test; Fig. 1c–d). Therefore, we defined a DCC methylation of 5 % or more as a continuous variable (i.e., >5.0 % methylation was defined as methylation-positive (methylated) and <5.0 % methylation as methylation-negative (unmethylated)). Using this criterion, we observed DCC methylated cases in 44/98 gastric cancers (45 %) and in 9/105 normal gastric mucosa (9 %).

Next, we investigated the association between DCC promoter methylation and various clinicopathological and genetic features. DCC methylation status was significantly associated with MSI status. MSI-positive gastric cancers were significantly more frequently associated with DCC methylation than with DCC unmethylation (23 vs. 6 %, P = 0.013; Table 2). There were no significant associations between DCC methylation status and any other variables.
A

Chromosome 18

D18S35  D18S69  D18S64

50.00Mb  52.00Mb  54.00Mb  56.00Mb  58.00Mb  60.00Mb

DCC

B

C

Methylation Density (%)

NM (n=31) T (n=56)

Population of Subjects Defined as Methylated (%)

NM  T

E

Fig. 1 (See legend on next page.)
LOH of 18q locus associated with CIN phenotype in gastric cancer

Among informative cases, the frequencies of 18q LOH at each microsatellite marker were 14/41 (24 %) at D18S535, 17/55 (31 %) at D18S569, and 21/58 (36 %) at D18S558 (the location of each maker is shown in Fig. 1a). Tumors showing LOH at all three, two, and only one of the three microsatellite markers were 4 (4.8 %), 9 (11 %), and 23 (28 %) of 83 informative cases among 98 primary gastric cancers, respectively. Tumors showing LOH in at least one of the three microsatellite markers for 18q LOH were categorized as 18q LOH-positive. By this criterion, 18q LOH-positive cancers were detected in 36 (43 %) of 83 informative cases among 98 primary gastric cancers (Table 2).

Similarly to the DCC methylation status, we investigated associations between 18q LOH status and various clinicopathological and genetic features. Among informative cases, the frequency of gastric cancer with distant metastases was higher in 18q LOH-positive gastric cancers compared with 18q LOH-negative cancers (25 vs. 9 %, \( P = 0.0041 \); Table 2). The LOH ratios calculated for the other seven loci were also significantly higher for 18q LOH-positive than for 18q LOH-negative tumors (0.44 vs. 0.10, \( P < .0001 \); Table 2). According to these results, when a tumor showed a LOH ratio higher than 0, the tumor was categorized as CIN-positive. Furthermore, CIN-positive gastric cancers that were also 18q LOH-positive were significantly more abundant than those that were 18q LOH-negative (74 vs. 38 %, \( P = 0.0017 \); Table 2). Previous studies have demonstrated that 18q loss is commonly observed in colorectal cancers, and its frequency is correlated with CIN phenotype but inversely correlated with MSI phenotype [29, 30]. As our study and another study demonstrated, this phenomenon was also reproduced for gastric cancers [18].

Expression and methylation status of DCC in gastric cancer cell lines

To assess associations between DCC expression status and epigenetic alterations in the DCC gene, we examined messenger RNA (mRNA) levels by reverse transcription quantitative polymerase chain reaction (RT-qPCR) using a primer set previously described [31] and examined associations between DCC expression and CpG methylation status in the DCC promoter region in 10 gastric cancer cell lines (MKN7, N87, MKN74, MKN45, NUGC-2, NUGC-3, NUGC-4, GCIY, OGUM-1, and MKN1) and one normal lung fibroblast cell line (NHHLF). All cell lines, except for MKN74, GCIY, OGUM-1, MKN1, and NHHLF cells, showed decreased expression of DCC gene transcripts, and their promoters were methylated (Fig. 1e). On the other hand, although three cell lines, N87, OGUM-1, and MKN1 showed decreased expression of DCC gene transcripts, DCC promoters in those cells were not methylated by COBRA. Among the 10 gastric cancer cell lines, only the GCIY cell line expressed DCC transcripts at the same level as NHHLF cells, but its promoter was methylated. When we categorized a cell line showing aΔC_T of more than 15.0 as DCC expression-negative and a cell line with aΔC_T of 15.0 or less as DCC expression-positive, only the GCIY cell line could be categorized as DCC expression-positive among the seven gastric cancer cell lines with DCC methylation; this finding was statistically non-significant.

Reduction of DCC expression requires both genetic and epigenetic alterations

Next, we investigated the association between DCC protein expression and genetic and epigenetic alterations in the DCC gene in 86 gastric cancer specimens. Representative examples of immunohistochemistry (IHC) staining results are shown in Fig. 2a–c. We categorized tumors into the following three groups based upon the IHC results analyzed as a categorical variable: complete loss of DCC expression (8 cases, 9 %; Fig. 2a), focal loss of DCC expression (38 cases, 44 %; Fig. 2b), and positive DCC protein expression (40 cases, 47 %; Fig. 2c). Among 86 gastric cancers, 46 cases (53 %) displayed reduced DCC expression. A previous study reported that reduced DCC expression was observed in a total of 38 % of gastric cancers, and stage T1–T2 tumors maintained a positive DCC expression while it was abolished in T3 tumors [32]. This finding was also reproducible in this study (Additional file 2: Figure S2).
Table 2  Association between epigenetic/genetic alterations of the DCC gene and clinicopathological features in gastric cancers

|                          | DCC Methylation Status—% (No.) | 18q LOH Status—% (No.) | DCC Alteration Status—% (No.) | P       |
|--------------------------|--------------------------------|------------------------|-------------------------------|---------|
|                          | Unmethylation                  | Methylation            | Not informative               | Positive |         |         |
|                          | (n = 54)                       | (n = 44)               | (n = 15)                      | (n = 36) |         |         |
| Age                      | Mean age (SD)                  | 63.6 (12.5)            | 67.0 (10.7)                   | 0.48*    |         |         |
| Gender                   | Female                         | 39 (21)                | 30 (13)                       | 0.33*    |         |         |
|                          | Male                           | 61 (23)                | 70 (31)                       | 0.21     |         |         |
| Histology                | Diff                           | 41 (22)                | 59 (26)                       | 0.071b   |         |         |
|                          | Undiff                         | 59 (32)                | 41 (18)                       | 0.071b   |         |         |
| Stage                    | IA/IB                          | 20 (11)                | 16 (7)                        | 0.83*    |         |         |
|                          | IIA/IIB                        | 26 (14)                | 34 (15)                       | 0.15b    |         |         |
|                          | IIIA/IIIB/IC                   | 39 (21)                | 36 (16)                       | 0.76b    |         |         |
|                          | IV                             | 15 (8)                 | 14 (6)                        | 0.24b    |         |         |
|                          | T1a/1b                         | 15 (8)                 | 14 (6)                        | 0.94b    |         |         |
|                          | T2                             | 13 (7)                 | 16 (7)                        | 0.155    |         |         |
|                          | T3                             | 20 (11)                | 36 (16)                       | 0.39     |         |         |
|                          | T4a/b                          | 52 (28)                | 34 (15)                       | 0.041b   |         |         |
|                          | N0                             | 30 (16)                | 23 (10)                       | 0.29b    |         |         |
|                          | N1                             | 37 (20)                | 32 (14)                       | 0.44     |         |         |
|                          | N2                             | 20 (11)                | 30 (13)                       | 0.49     |         |         |
|                          | N3                             | 13 (7)                 | 16 (7)                        | 0.089    |         |         |
| Distant metastasis       | Negative                       | 85 (46)                | 86 (38)                       | 0.87*    |         |         |
|                          | Positive                       | 15 (8)                 | 14 (6)                        | 0.94     |         |         |
| MSI status               | MSI                            | 6 (3)                  | 23 (10)                       | 0.013b   |         |         |
|                          | Non-MSI                        | 94 (51)                | 77 (34)                       | 0.17b    |         |         |
| LOH ratio                | Mean ratio (SD)                | 0.23 (0.30)            | 0.26 (0.31)                   | 0.55*    |         |         |
| CIN*                     | Positive                       | 46 (25)                | 57 (25)                       | 0.39*    | <0.0001*|         |
|                          | Negative                       | 54 (27)                | 43 (19)                       | 0.007b   |         |         |
| KRAS/BRAF/PIK3CA         | Wild                           | 63 (31)                | 30 (13)                       | 0.30*    | <0.0001*|         |
|                          | Positive                       | 72 (39)                | 70 (31)                       | 0.85*    |         |         |
|                          | Negative                       | 28 (15)                | 30 (13)                       | 0.07*    |         |         |

*a Two cases are not informative of CIN status

*b P value were calculated between unmethylation and methylation, 18qLOH negative and positive, and DCC alteration negative and positive(total) by Wilcoxon/Kruskal–Wallis test

*b P values were calculated between unmethylation and methylation, 18qLOH negative and positive, and DCC alteration negative and positive(total) by Piason's chi-square test

| Stage | IA/IB | IIA/IIB | IIIA/IIIB/IC | IV | T | T1a/1b | T2 | T3 | T4a/b | N0 | N1 | N2 | N3 | Distant metastasis | Negative | Positive | MSI | Non-MSI | LOH ratio | CIN* | KRAS/BRAF/PIK3CA | Wild | Positive | Negative | H.pylori | Positive | Negative |...
|-------|-------|---------|--------------|----|---|-------|----|----|-------|----|----|----|----|---------------------|---------|----------|-----|---------|-----------|------|-----------------|------|----------|----------|---------|----------|----------|...
Next, we examined associations between genetic and epigenetic alterations and DCC expression status. Seven of 8 cases with complete loss, 33 of 38 cases with focal loss, and 34 of 40 cases with positive DCC expression were informative for both DCC promoter methylation and 18q LOH. We found that among the cancers with complete loss of DCC expression, 5/7 cancers (71%) demonstrated both DCC promoter methylation and 18q LOH. In contrast, 8/33 cancers (24%) showed focal loss of DCC expression and both methylation and LOH, 5/33 cancers (15%) showed LOH alone, and 12/33 cancers (36%) displayed methylation alone. Among the cancers that were positive for DCC expression, only 2/34 cancers (6%) demonstrated both methylation and LOH, 13/34 (38%) cancers LOH alone, and 8/34 cancers (24%) methylation alone (cancers showing both DCC methylation and 18q LOH vs. the others, $P = 0.0048$, Pearson’s chi-square test; Fig. 2d). Our data suggest that a reduction in DCC expression may require dense methylation in the promoter CpGs and LOH of the 18q locus, according to the two-hit theory [33].

### Figures

**Figure 2**

**A** DCC promoter methylation and immunohistochemistry analyses. Immunohistochemistry analysis for DCC (a–c). Nuclei of tumor cells are completely negatively (a), focally negatively (b), and positively (c) stained.

**B** Association between epigenetic/genetic alteration and DCC expression.

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**Association between clinicopathological features and genetic/epigenetic alterations of DCC in gastric cancer**

Since both epigenetic and genetic alterations are critical to DCC suppression, we investigated the relationship between epigenetic and genetic alterations in the DCC gene with various clinicopathological features. Of 98 gastric cancers, 15 cancers were categorized as non-informative, 25 cancers were categorized as negative for DCC alterations, and 58 cancers were categorized as positive for DCC alterations. Among clinicopathological features, LOH ratio and CIN phenotype distribution differed significantly between cancers negative and positive for DCC alterations (Table 2).

Among the 58 cancers with DCC alterations, 17 tumors showed alterations in both DCC methylation and
Methylation profiles of UNC5C in gastric cancer specimens

UNC5C methylation status was examined in a cohort of 98 gastric cancers and 105 normal gastric mucosa specimens. Location of the UNC5C gene and a panel of representative COBRA results are depicted in Fig. 3a–b. We analyzed these results using UNC5C methylation levels as continuous variables. We found that 39/98 gastric cancers (40 %) and 16/105 normal gastric mucosa specimens (15 %) displayed more than 1 % methylation in the UNC5C promoter. Of the samples that showed more than 1 % methylation in the UNC5C promoter, mean methylation levels of UNC5C were significantly higher in gastric cancers compared with their corresponding normal gastric mucosa specimens (17.4 % (95 % CI, 12.4–22.4 %) in gastric cancers; 6.3 % (95 % CI, 3.1–9.5 %) in normal gastric mucosa specimens; \( P < 0.0001 \), Wilcoxon/Kruskal–Wallis test; Fig. 3c–d). Using these results, we optimized a cutoff value of UNC5C methylation of 5 % (>5 % methylation as positive and ≤5 % methylation as negative). Using this cutoff value, 31/98 gastric cancers (32 %) and 5/105 normal gastric mucosa specimens (5 %) were diagnosed as UNC5C-methylated. Next, we examined associations between UNC5C promoter methylation status and the clinicopathological and genetic features of gastric cancers. UNC5C methylation showed a significant association with MSI status. MSI-positive gastric cancers were significantly more frequent in gastric cancers with UNC5C methylation compared with those without UNC5C methylation (26 vs. 7 %, \( P = 0.013 \); Table 3). There were no significant associations between UNC5C methylation status and any of the other variables.

Expression and methylation status of UNC5C in gastric cancer cell lines

Before examining UNC5C expression and methylation status in the UNC5C promoter region in the gastric cancer cell lines, we assessed the expression status of five splicing variants of UNC5C mRNA (UNC5C-001, 002, 003, 004, and 201) by RT-PCR (Additional file 3: Figure S3). When analyzing five splicing variants of UNC5C messenger RNA in 10 gastric cancer cell lines (MKN7, N87, MKN74, MKN45, NUGC-2, NUGC-3, NUGC-4, GCIY, OGUML-1, and MKN1) and one normal lung fibroblast cell line (NHFL), we found that only two cell lines, GCIY and NHFL, expressed all five splicing variants of UNC5C messenger RNA. In nine gastric cancer cell lines, there was no expression of any of the five splicing variants of UNC5C mRNA (Additional file 4: Figure S4). Thus, further examination of mRNA expression levels by RT-qPCR was performed using the UNC5C 001–004 primer set. Among the 10 gastric cancer cell lines, only the GCIY cell line exhibited increased UNC5C mRNA expression levels (lower \( \Delta C_T \), Fig. 3e). When we categorized UNC5C mRNA expression status by RT-PCR results (Additional files 4 and 5: Figures S4 and S5), 93 % (13/14) of UNC5C methylated cell lines lacked UNC5C mRNA expression, whereas 33 % (1/3) of UNC5C unmethylated cell lines lacked UNC5C mRNA expression (\( P = 0.01 \), Pearson’s chi-square test). Thus, UNC5C gene transcript expression was significantly associated with promoter methylation level. Additionally, we tried to perform IHC staining for UNC5C protein expression. Unfortunately, we were unable to analyze UNC5C protein expression due to lack of appropriate antibodies.

LOH of the 4q locus associated with CIN phenotype in gastric cancer

The frequencies of 4q21–23 LOH at each microsatellite marker for UNC5C were 29 % (11/38 informative cases) at D4S2380, 23 % (10/44) at D4S470, and 28 % (12/43) at D4S1559 (location of each marker is shown in Fig. 3a). Tumors showing LOH at all three, two, and only one of the three microsatellite markers amounted to 3 (3.8 %), 5 (6.3 %), and 15 (20 %) of 79 informative cases among 98 primary gastric cancers, respectively. We defined 4q LOH-positive tumors as those showing LOH on at least one of the three microsatellite markers. Tumors showing 4q LOH were found in 23 (29 %) of 79 informative cases among 98 primary gastric cancers (Table 3). We found that differentiated adenocarcinomas were significantly more frequently observed in cancers with 4q LOH (65 % in 4q LOH-positive cancers vs. 39 % in 4q LOH-negative ones, \( P = 0.036 \)).
Fig. 3 (See legend on next page.)
Similar to 18q LOH, we found a strong correlation between 4q LOH and CIN phenotype. The LOH ratio calculated for the remaining seven loci was significantly higher in gastric cancers with 4q LOH compared to those without 4q LOH (0.40 vs. 0.15, \( P < 0.0001 \); Table 3). According to these results, when a tumor showed a LOH ratio higher than 0, the tumor was categorized as CIN-positive, and CIN-positive gastric cancers were significantly more frequent in the presence of 4q LOH (83% in 4q LOH-positive tumors vs. 37% in 4q LOH-negative ones, \( P = 0.0003 \); Table 3). While there were no significant associations among any of the other variables, all KRAS/PIK3CA mutations were found in 4q LOH-negative gastric cancers with a non-significant difference (\( P = 0.08 \)).

**Association between clinicopathological features and genetic/epigenetic alterations of UNC5C in gastric cancer**

Next, we investigated the relationship between UNC5C alterations and clinicopathological features. Of 98 gastric cancers, 19 cancers were categorized as non-informative, 39 cancers were categorized as negative for UNC5C alterations, and 40 cancers as positive for UNC5C alterations. Cancers with UNC5C alterations were more frequently observed in advanced stages (37% (14/38) for stages I and II vs. 63% (26/41) for stages III and IV, \( P = 0.02 \), Pearson's chi-square test) and in advanced stages for lymph node metastasis (25% (5/20) for N0 vs. 59% (35/59) for N1–N3, \( P = 0.008 \), Pearson’s chi-square test).

Among the 40 cancers with UNC5C alterations, 10 cancers showed alterations both in terms of UNC5C methylation and 4q LOH, 13 cancers showed 4q LOH alone, and 17 cancers showed UNC5C methylation alone. The LOH ratio calculated for the remaining seven loci was significantly higher in cancers with both UNC5C methylation and 4q LOH (mean LOH ratio, 0.45; SD ±0.36) and in those with 4q LOH alone (0.37 ± 0.25), intermediate in cancers with UNC5C methylation alone (0.24 ± 0.29), and lowest in cancers negative for UNC5C alterations (0.11 ± 0.22, \( P = 0.0002 \), Wilcoxon/Kruskal–Wallis test). When we categorized the cancers that showed LOH ratios higher than 0 as CIN-positive, 8/10 gastric cancers (80%) with both UNC5C methylation and 4q LOH, 11/13 gastric cancers (85%) with 4q LOH alone, 9/17 gastric cancers (53%) with UNC5C methylation alone, and 11/39 cancers (30%) negative for UNC5C alterations were categorized as CIN-positive (\( P = 0.001 \), Pearson’s chi-square test). Similar to the DCC alterations in gastric cancer, our data highlight that UNC5C alterations caused by both epigenetic and genetic events were significantly associated with CIN-positive gastric cancers.

**Cumulative loss of netrin-1 receptors accrues with gastric cancer progression**

Because UNC5C and DCC both serve as dependence receptors for netrin-1, we investigated whether defects in these receptors accumulate in a systematic or stochastic manner during the progression of gastric carcinoma. Therefore, we looked for associations between UNC5C and/or DCC defects and TNM stage in the 98 gastric cancers that were informative for both UNC5C and DCC genetic/epigenetic results (Fig. 4 and Additional file 6: Table S1). Concurrent alterations in the DCC and UNC5C genes were observed significantly more commonly in advanced stages (64% (21/33) for stages III and IV) than in earlier-stage cancers (24% (8/33) for stages I and II, \( P = 0.001 \), Pearson’s chi-square test; Fig. 4a). By stratifying gastric cancers based on individual defects in either UNC5C or DCC and their relationship with tumor stage, UNC5C alterations were found in 18% (2/11 informative cases), 48% (12/25 informative cases), 63% (19/30 informative cases), and 64% (7/11 informative cases) of stages I, II, III, and IV cancers, respectively, showing that UNC5C alterations gradually developed according to the progression of the TNM stage (Table 3). On the other hand, DCC alterations were constantly observed with a high frequency in all TNM stages; hence, DCC alterations were found in 63% (10/16 informative cases), 76% (19/25 informative cases), 66% (19/29 informative cases), and 77% (10/13 informative cases) of stages I, II, III, and IV cancers, respectively (Table 2). Therefore, this differential feature found for each gene taken into consideration indicates that cumulative alterations of netrin-1 receptors are...
Table 3 Association between epigenetic/genetic alterations of the UNC5C gene and clinicopathological features in gastric cancers

| UNC5C Methylation Status—% (No.) | 4q LOH Status—% (No.) | UNC5C Alteration Status—% (No.) | P |
|---------------------------------|----------------------|---------------------------------|---|
| Unmethylation                   | Methylation          | Not informative                 |   |
| (n = 67)                        | (n = 31)             | (n = 19)                        |   |
| Age                             | Mean age (SD)        | 64.3 (12.6)                     |   |
| Gender                          |                      | 66.7 (9.7)                      |   |
| Male                            |                      | 65.4 (12.3)                     |   |
| Histology                       |                      | 68.3 (8.2)                      |   |
| Stage                           |                      | 60.2 (12.6)                     |   |
| T                              |                      | 64.2 (9.7)                      |   |
| N                               |                      | 66.7 (9.7)                      |   |
| Positive                        |                      | 65.4 (12.3)                     |   |
| T1a/1b                          |                      | 68.3 (8.2)                      |   |
| IIa/IIb                         |                      | 60.2 (12.6)                     |   |
| Undiff                          |                      | 65.4 (12.3)                     |   |
| IV                              |                      | 68.3 (8.2)                      |   |
| Stage                           |                      | 64.2 (9.7)                      |   |
| T                               |                      | 65.4 (12.3)                     | 0.17 |
| N                               |                      | 68.3 (8.2)                      | 0.19 |
| Positive                        |                      | 60.2 (12.6)                     | <0.0001 |
| T1a/1b                          |                      | 64.2 (9.7)                      | 0.49 |
| IIa/IIb                         |                      | 65.4 (12.3)                     | <0.0001 |
| Undiff                          |                      | 68.3 (8.2)                      | 0.36 |
| IV                              |                      | 60.2 (12.6)                     | 0.36 |
| Distant metastasis              |                      | 65.4 (12.3)                     | 0.24 |
| Positive                        |                      | 68.3 (8.2)                      | 0.37 |
| MSI                             |                      | 60.2 (12.6)                     | 0.37 |
| Non-MSI                         |                      | 64.2 (9.7)                      | 0.37 |
| LOH Ratio                       | Mean ratio (SD)      | 65.4 (12.3)                     | 0.37 |
| CIN*                            |                      | 68.3 (8.2)                      | 0.37 |
| Positive                        |                      | 60.2 (12.6)                     | 0.37 |
| Negative                        |                      | 64.2 (9.7)                      | 0.37 |

*Two cases are not informative of CIN status

**P** value were calculated between unmethylation and methylation, 4qLOH negative and positive, and UNC5C alteration negative and positive (total) by Wilcoxon/Kruskal–Wallis test

**P** values were calculated by Pearson’s chi-square test
Alterations found in Both Receptors (n=29)
Either Receptor (n=22)
Neither Receptor (n=15)

P=0.001 *

Alterations in NTN1 receptor

LOH Ratio

I (n=11) II (n=22) III (n=23) IV (n=10)

T1a/1b (n=9) T2 (n=10) T3 (n=13) T4a/4b (n=28)

Stage

N0 (n=17) N1 (n=20) N2 (n=19) N3 (n=10)

T Factor

Fig. 4 (See legend on next page.)
associated with gastric cancer progression. With respect to the factors that determine TNM classification, a cumulative loss of netrin-1 receptors was more strongly associated with the degree of regional lymph node metastasis (N factor, Fig. 4e) compared with the tumor status of T factors (Fig. 4b). Interestingly, gastric cancers with defects in either UNC5C or DCC did not show distant metastasis (Fig. 4e), suggesting that the cumulative alterations of netrin-1 receptors was a late event in gastric cancer progression, significantly associated with CIN-positive gastric cancers through increasing the LOH ratio (Fig. 4d, g) rather than MSI and mutational status (Fig. 4f). On the other hand, there were no significant associations between cumulative loss of netrin-1 receptors and any other clinicopathological variables (Additional file 7: Figure S6).

### Discussion

This study investigated the molecular events responsible for the abrogation of the netrin pathway in gastric cancer and the role played by the two dependence receptors, DCC and UNC5C. We analyzed 98 gastric cancers and 105 adjacent normal mucosa specimens. We found that the frequency of gastric cancers with concurrent alterations in the DCC and UNC5C genes increased in a stage-dependent manner. Upon stratifying gastric cancers based on defects in either DCC or UNC5C and on their relationship with tumor stage, we found that DCC alterations were consistently observed in all TNM stages with a high frequency: 10 of 16 (63 %) of stage I, 19 of 25 (76 %) of stage II, 19 of 29 (66 %) of stage III, and 10 of 13 (77 %) of stage IV cancers (Table 2). Meanwhile, UNC5C alterations gradually increased according to the progression of the TNM stage and were found in 2/11 (18 %) of stage I, 12 of 25 (48 %) of stage II, 19 of 30 (63 %) of stage III, and 7 of 11 (64 %) of stage IV cancers (Table 3). Both DCC and UNC5C were inactivated in 97 % of CIN-positive gastric cancers and in 55 % of CIN-negative gastric cancers, and these alterations occurred through genetic and epigenetic processes. These data provide novel evidence that the timing of molecular alterations in DCC and UNC5C is not random, because DCC inactivation occurs through all tumor stages, whereas UNC5C inactivation accrues gradually during multistep gastric carcinogenesis.

Cells expressing netrin-1 receptors can send a survival signal when they are engaged by netrin-1. On the other hand, these receptors will send a death signal when they are disengaged [34]. Thus, a loss of netrin-1 receptors on tumor cells represents a loss of dependence receptors that are capable of mediating apoptosis, resulting in enhanced tumor cell survival [35].

The netrin-1 receptor, DCC, was discovered as a putative tumor suppressor gene in colorectal cancer [21]. DCC is located on chromosome 18q, which is the most common deleted chromosomal region in colorectal cancer as well as gastric cancer [13, 36–38, 12, 14]. The tumor-suppressor role for DCC has been questioned in studies that failed to show a clear malignant phenotype in DCC knockout mouse models [20]. However, recent studies have also challenged this hypothesis and have suggested a role for DCC in suppressing tumor growth and metastasis [24, 25]. Recent indications that DCC serves as a dependence receptor for netrin-1 have renewed the hypothesis that DCC functions as a pro-apoptotic growth suppressor when not bound to its ligand [34, 39, 12, 10]. In the gastrointestinal tract, netrin-1 has an important role in the maintenance and renewal of the intestinal epithelium by regulating cell survival or cell death through its interaction with its receptors, DCC and UNC5C [24, 34, 39]. In line with previous studies [11, 8], in this study, we demonstrated that methylation-induced silencing of DCC as well as allelic loss of 18q was critical to loss of DCC expression. Thus, reduction of DCC expression may require dense CpG promoter methylation and LOH of the 18q locus according to the two-hit theory as a common behavior of tumor suppressor genes [33].

The other netrin-1 receptors, UNC5A, UNC5B, and UNC5C, were also discovered as putative tumor suppressor genes in various tumors [26, 27]. Among them, a twofold downregulation of UNC5C expression compared with corresponding normal tissue was observed in approximately 70 % of gastric cancer cases [26]. Therefore, we focused on UNC5C that may play a more critical role for gastric carcinogenesis. Additionally, it was suggested...
that the loss of UNC5C was caused by allelic losses of chromosome 4q, and mutations were rarely observed [26]. Allelic losses at the 4q locus have been reported previously in several human cancers, with frequencies ranging from 23–39 % [26, 32, 18]. In accordance with previous studies, in the present study, the frequencies of allelic loss at 4q21–23 LOH were 29 % (11/38 informative cases) on D4S2380, 23 % (10/44) on D4S470, and 28 % (12/43) on D4S1559. Finally, gastric cancers demonstrating allelic loss at 4q were found in 29 % (23/79) of the informative cases. Another mechanism underlying the loss of UNC5C in human cancers is represented by epigenetic alterations. Indeed, we previously reported that UNC5C was silenced by dense methylation of its promoter CpG islands in colorectal cancer [28]. As is the case in colorectal cancer, our results demonstrated that 11/12 gastric cancer cell lines (Fig. 3e and Additional files 3 and 4: Figures S3–S4) lacking UNC5C expression showed dense methylation in the UNC5C promoter. In clinical specimens, 31/98 gastric cancers (32 %) and 5/105 normal gastric mucosa specimens (5 %) exhibited UNC5C methylation. Therefore, we performed UNC5C IHC on the clinical specimens to examine the two-hit theory in which aberrant promoter methylation and allelic losses were the key factors determining lack of DCC. However, we were unable to perform UNC5C IHC on the clinical specimens because of the lack of an appropriate antibody for tissue staining.

Because both DCC and UNC5C share the same netrin ligand and are colocalized in the gut [24, 23, 26, 40], we hypothesized that solitary inactivation of either DCC or UNC5C may not be sufficient to promote tumor development in the stomach. In this study, we found that 97 % of gastric cancers with CIN and 55 % of those without CIN showed simultaneous alterations in both DCC and UNC5C, supporting our hypothesis that inactivation of both receptors may be required in the development of gastric cancer. Our finding that dysregulation of DCC predominantly occurs in the early phase of gastric cancer whereas UNC5C alterations occur later suggests that inactivation of these receptors is not a random process but occurs in a statistically predictable, sequential manner.

H. pylori in the human gastric mucosa is a well-known inducer of chronic inflammation and gastric cancers and is associated with a high incidence of aberrant DNA methylation [7, 41, 30, 29]. So, we detected H. pylori infection by recovering the cagA repeat sequence from gastric cancer specimens as well as normal gastric mucosa samples. Of 98 cancers, 70 cancers were positive for the cagA sequence. However, there was no association between the presence of the cagA sequence in cancer tissue and clinicopathological and molecular features, specifically not DCC nor UNC5C methylation incidence. Conversely, with respect to normal counterpart gastric mucosa, a total of 102 normal gastric mucosa samples were available for analyzing the presence of the cagA sequence in this study. Of 102 gastric mucosa samples, the cagA sequence was successfully recovered in 79 (74 %). Interestingly, when we defined DCC and UNC5C methylation at 1 % or more as a continuous variable (i.e., >1.0 % methylation as methylation-positive (methylated) and <1.0 % methylation as methylation-negative (unmethylated)), only UNC5C methylation was significantly associated with the presence of the cagA sequence in normal gastric mucosa (data not shown), suggesting that inflammatory processes associated with H. pylori infection causes aberrant methylation in UNC5C promoter CpGs but not in the DCC promoter. Thus, our results suggested that H. pylori infection does not induce aberrant methylation randomly but in target loci by a particular signal cascade. To address questions underlying H. pylori infection and epigenetic changes, further investigations are warranted.

Conclusions
We provide previously unrecognized and novel evidence that most gastric cancers, particularly those with CIN, possess alterations in both DCC and UNC5C receptors. Such alterations are apparent in the early stages and continue to escalate in both receptor types with disease progression, emphasizing the importance of this growth regulatory pathway in gastric carcinogenesis.

Methods
Primary gastric specimens
We collected tissue specimens of primary gastric cancer and matched normal gastric mucosa from 105 patients who had undergone surgery at the Okayama University Hospital (Okayama, Japan). Of 105 gastric cancer patients, seven did not have sufficient tumor tissue for analysis. Thus, in this study, a total of 98 gastric cancer specimens and 105 matched normal gastric mucosa tissues were analyzed. All normal gastric mucosa specimens were obtained from sites adjacent to the tumor but at least 5 cm away from the tumor site. All patients provided written informed consent, and the study was approved by the ethics committee of the Okayama University Hospital. All patients also gave informed consent for usage of their data for future analyses. The histological diagnosis was established according to the World Health Organization International Histological Classification of tumors, with subclassification into two histological categories: differentiated type (well and moderately differentiated tubular adenocarcinoma) and undifferentiated type (poorly differentiated adenocarcinoma and mucinous adenocarcinoma). The pathological stage
was determined according to the International Union Against Cancer TNM classification (Seventh edition).

**Cell lines**

A total of 12 human gastric cancer cell lines (MKN7, N87, MKN74, MKN45, NUGC-2, NUGC-3, NUGC-4, GCIY, OGUM-1, MKN1, MKN28, and KATO III), three human colorectal cancer cell lines (SW48, SW480, and SW837), one human colon fibroblast cell line (CCD18Co), and one human lung fibroblast cell line (NHLF) were purchased from American Type Culture Collections (Manassas, VA, USA), Japanese Collection of Research Bioreresources Cell Bank (Ibaragi, Osaka, Japan), Health Science Research Resources Bank (Chiyoda-ku, Tokyo, Japan), RIKEN (Wako, Saitama, Japan), or Cambrex Bio Science Walkersville, Inc. (East Rutherford, NJ, USA). All cell lines were cultured in appropriate culture medium supplemented with 10 % fetal bovine serum, penicillin (100 IU/mL), and streptomycin (100 μg/mL) at 37 °C in a humidified incubator with 5 % CO₂.

**DNA and RNA extraction**

Genomic DNA from the cell lines was extracted using QIAamp DNA Mini Kit (Qiagen, Valencia, CA, USA). All gastric cancers and normal gastric mucosa samples were fresh-frozen tissue specimens, from which DNA was extracted using standard procedures that included proteinase-K digestion and phenol–chloroform extraction. Total RNA from six cultured cell lines (MKN28, KATO III, SW48, SW480, SW837, and CCD18Co) was obtained using the TRIzol reagent (Invitrogen Life Technologies Inc., Carlsbad, CA, USA). Total RNA from 10 gastric cancer cell lines (MKN7, N87, MKN74, MKN45, NUGC-2, NUGC-3, NUGC-4, GCIY, OGUM-1, and MKN1) and a normal lung fibroblast cell line, NHLF, was isolated using the RNeasy Mini Kit (Qiagen) according to the manufacturer’s instructions.

**Reverse transcription polymerase chain reaction**

The first-strand complementary DNA synthesis was performed using the Moloney murine leukemia virus reverse transcriptase (Invitrogen Life Technologies Inc.) and miScript II RT Kit (Qiagen) with a total of 1.0 μg RNA. RT-PCR was performed using specific primer pairs for UNC5C (including primers for the detection of splicing variants), DCC, and beta-actin (Additional file 8: Table S2). The PCR products were electrophoresed on a 3.5 % agarose gel. By using the LINC5C 001–004-, DCC-, and beta-actin-specific primer pairs, expression of LINC5C and DCC mRNA was also determined by RT-qPCR using the SsoAdvanced Universal SYBR Green Supermix on the LightCycler 480 (Roche Diagnostics). The expression level of each target gene was analyzed based on the ΔC_T method, and beta-actin was used as an endogenous control to normalize the amount of total RNA in each sample.

**Bisulfite modification and combined bisulfite restriction analysis**

Bisulfite modification of genomic DNA from cell lines and clinical specimens was performed as described previously. The methylation status of the DCC and LINC5C promoters in gastric tissues and cell lines was analyzed by combined bisulfite restriction analysis (COBRA, Additional file 8: Table S2). COBRA was carried out in a 24.0-μL PCR reaction containing 12.0 μL of HotStarTaq Master Mix (Qiagen) and 0.4 μM of each primer. PCR products were digested with a restriction enzyme Hhal (New England Biolabs Inc., Ipswich, MA, USA) at 37 °C overnight. PCR products were electrophoresed on a 3.0 % agarose gel. The percentage of methylated Hhal sites were calculated by determining the ratio between the Hhal-cleaved PCR product and the total amount of PCR product loaded.

**LOH analyses and definition of CIN phenotype**

A set of three polymorphic microsatellite markers per gene was used to determine LOH at chromosomes 18q21 for DCC and 4q21–23 for LINC5C (Additional file 8: Table S2). PCR amplifications were performed on genomic DNA templates from both tumor and normal mucosa tissue using fluorescently labeled primers. PCR products were electrophoresed on an ABI 310R Genetic Analyzer and analyzed by GeneMapper fragment analysis software (Applied Biosystems, Foster City, CA, USA). When comparing the signal intensities of the individual markers in the tumor DNA with that of the corresponding normal DNA, a reduction by at least 40 % of the signal intensity was considered indicative of LOH.

In addition, to examine the association between netrin-1 receptor disorders and CIN phenotype, we analyzed additional seven sets of polymorphic microsatellite sequences that are tightly linked to known tumor suppressor genes and DNA mismatch repair genes, including the MYCL locus on 1p34 (MYCL), the hMSH2 locus on 2p16 (D2S123), the APC locus on 5q21 (D5S346, D5S107), the LINC5D locus on 8p12 (D8S87), and the p53 locus on 17p13 (D17S250, TP53) [31]. Of 98 gastric cancer patients, 96 patients displayed at least one marker informative of LOH status. Hence, since two patients turned out non-informative for LOH at all seven microsatellite sequences, further analyses were performed only on the 96 informative cases. CIN phenotype classification was performed by calculating the LOH ratio of the informative markers of the seven polymorphic microsatellite sequences, independently from
the LINCS and DCC loci. When a tumor showed a LOH ratio higher than 0, the tumor was categorized as CIN-positive.

**MSI analysis and definition of MSI phenotype**

MSI status was analyzed for all 98 gastric cancer patients using three mononucleotide repeat markers (BAT26, NR21, and NR22) as described previously [33]. When at least one or more mononucleotide repeat markers displayed microsatellite instability, tumors were defined to have an MSI phenotype, and tumors without MSI in the three mononucleotide repeat markers were defined to have a non-MSI phenotype.

**KRAS, BRAF, and PIK3CA mutation analyses**

KRAS and BRAF mutation status was analyzed in all 98 patients as described previously [35]. In addition, PIK3CA exon 9 and 20 mutation status was also analyzed by direct sequencing. PCR and sequencing were performed using PIK3CA exon 9- and 20-specific primer pairs (Additional file 8: Table S2). PCR products were electrophoresed on an ABI 310R Genetic Analyzer.

**Detection of H. pylori**

To determine H. pylori infection status, we recovered the Glu-Pro-Ile-Tyr-Ara (EPIYA) repeat sequence in the cagA protein, which binds to the Src homology 2 domain-containing protein tyrosine phosphatase, SHP-2, on gastric epithelial cells. The cagA was recovered by PCR amplification performed on genomic DNA templates from tumor tissues. We modified the primer design to develop PCR products shorter than the PCR products described previously [42, 43]. PCR was carried out in a 24-μL PCR reaction containing 12 μL of HotStarTaq Master Mix and 0.4 μM of each primer (Additional file 8: Table S2). The PCR products were electrophoresed on a 3 % agarose gel.

**Immunohistochemical analysis**

A total of 89 gastric cancers from 98 patients were available for IHC staining for DCC protein expression analysis. Staining was carried out manually with formalin-fixed paraffin-embedded tissues. Thin (5 μm) sections of representative blocks were deparaffinized and dehydrated using gradient solvents. Following antigen retrieval in citrate buffer (pH 6.0), endogenous peroxidase was blocked with 3 % H2O2. Thereafter, slides were incubated overnight in the presence of a purified mouse anti-human DCC monoclonal antibody (clone G97–449, Pharmingen, San Diego, CA, USA; dilution 1:100). A further incubation was carried out with a secondary antibody and the avidin-biotin-peroxidase complex (Vector Laboratories, Burlingame, CA, USA) and then incubated with biotinyltyramide followed by streptavidin-peroxidase. Diaminobenzidine was used as a chromogen, and hematoxylin as a nuclear counterstain. Tissue sections with obvious nuclear staining were considered positive. The only foci of neoplasia that were scored as negative were those for which there was definite evidence of unspacifically positively stained admixed or surrounding non-neoplastic cells such as normal colonic mucosal cells, lymphocytes, or stromal cells.

**Statistical analysis**

Statistical analyses were performed using JMP software (version 10.0; SAS Institute Inc., Cary, NC, USA). First, DCC and LINCS methylation levels were analyzed as continuous variables, as were computed means, standard errors of the means, and standard distributions. Next, the methylation status of the DCC and LINCS promoter was analyzed as a categorical variable (positive, methylation level >5 %; negative, methylation level <5 %). Differences in frequencies were evaluated by Pearson’s chi-square test or the Wilcoxon/Kruskal–Wallis test. Whenever the Kruskal–Wallis test indicated differences among these subgroups, further pairwise comparisons for each of the subgroup was performed by a non-parametric multiple comparison method using the Steel–Dwass test. All reported probability (P) values were two-sided, and a P value of less than 0.05 was considered statistically significant.

**Additional files**

- Additional file 1: Figure S1. Examples of KRAS mutation and H. pylori cagA analysis. (A) An example of KRAS codon 12 and 13 direct sequencing analysis. This case showed both codon 12 and 13 mutations. (B) To detect H. pylori infection, EPIYA repeat sequences in the cagA protein were recovered from clinical materials. We found two types of EPIYA repeat sequences, one was 159 bp and the other was 261 bp. SM denotes the size marker; P and N denote positivity and negativity of EPIYA repeat sequences, respectively.
- Additional file 2: Figure S2. Association between DCC IHC status and T factors.
- Additional file 3: Figure S3. Splicing variants of DCC mRNA and location of the primers which can distinguish expression status of the splicing variants.
- Additional file 4: Figure S4. Expression status of splicing variants of UNCSC mRNA and association of UNCSC methylation for 10 gastric cancer cell lines. Ten gastric cancer cell lines and NHLF cells were analyzed for mRNA expression by RT-PCR of UNCSC splicing variants and beta-actin genes. The lowest panel illustrates the methylation profile obtained from COBRA.
- Additional file 5: Figure S5. Association of UNCSC methylation and loss of UNCSC mRNA expression in gastric cancer and colorectal cancer cell lines. Two gastric cancer cell lines, three colorectal cancer cell lines, and CDD1BCCo cells were analyzed for mRNA expression by RT-PCR of UNCSC and beta-actin genes. The lowest panel illustrates the methylation profile obtained from COBRA.
- Additional file 6: Table S1. Association between alterations in netrin-1 receptors and clinicopathological features in gastric cancers.
- Additional file 7: Figure S6. Association between alteration patterns in netrin-1 receptors and clinicopathological features in gastric cancers.
Compelling interests

The authors declare that they have no competing interests.

Authors' contributions

KT performed methylation and LOH analyses of both genes in the clinical sample material and drafted the manuscript. TN assisted with the interpretation of the data, designed the project, secured the funding, and drafted the manuscript. YU and SK provided the patient samples and clinico-pathological data. TT performed IHC and collected pathological findings. NK and YT assisted with methylation and LOH analyses of both genes in the clinical sample material. HT and DS assisted with the methylation and LOH analyses in the cell lines and partially in the clinical sample material. TK and TomF performed RT-PCR and RT-qPCR strategies for DCC and UNC5C in the cell lines. FT and KY cultured all cell lines and examined the expression status of both genes with cell line work and IHC studies. AG assisted with the interpretation of the data, co-designed the project, and revised the manuscript. ToF provided patients' samples and clinico-pathological data, assisted with the interpretation of the data, and revised the manuscript. All authors read and approved the final manuscript.

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