Genetic and Epigenetic Alterations of Netrin-1 Receptors in Gastric Cancer with Chromosomal Instability

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Abstract

Background

The expression of the netrin-1 dependence receptors, *DCC* and *UNC5C*, is frequently downregulated in many cancers, including colorectal cancer. We hypothesized that downregulation of *DCC* and *UNC5C* has an important growth regulatory function in gastric tumorigenesis.

Results

In this study, a series of genetic and epigenetic analysis for *DCC* and *UNC5C* were performed in a cohort of 98 sporadic gastric cancers and corresponding normal gastric mucosa. Loss of heterozygosity (LOH) analyses and microsatellite instability (MSI) analysis determined chromosomal instability (CIN) and MSI phenotypes, respectively. Gastric cancers were categorized according to TNM stages (International Union Against Cancer 7th edition); 18, 29, 37, and 14 cases were stage I, II, III, and IV, 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 significantly associated with chromosomal instability.

Conclusions

The majority of gastric cancers harbor defects in netrin receptors. Such alterations are apparent in the early stages and continue to escalate in both receptors with the progression of the disease, thus emphasizing the importance of this growth regulatory pathway in gastric carcinogenesis.

Keyword

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

Background

Global estimates of cancer incidence ranked 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 etiologic risk factors for gastric cancer is *Helicobacter pylori* (*H. pylori*) infection. However, only a small proportion of individuals infected with *H. pylori* develop gastric cancer [4, 5]. It is widely known that gastric cancer results from a combination of environmental factors and the accumulation of generalized and specific genetic alterations, such as *p53* and *ARID1A* [6, 7]. In addition, recent studies have shown that epigenetic alterations (e.g., DNA methylation) play a key role in gastric cancer tumorigenesis [8, 9]. Understanding these alterations and the molecular mechanisms involved in gastric carcinogenesis is essential for the improvement of diagnosis, prognosis, and prediction of therapeutic outcomes of this disease.

With respect to genetic alterations, more than 60% of gastric cancers exhibit chromosomal instability (CIN) characterized by copy number changes in chromosomes [10]. 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 nonmalignant 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 regions included chromosomes 3p, 4p, 4q, 5q, 6q, 9p, 14q, 18q, and 21q [11].

Frequently deleted chromosomal regions were classically marked by loss of heterozygosity (LOH) and suggest the presence of tumor suppressor genes [12, 13]. LOH on chromosome 18q21 is frequently found in gastric cancers [11, 14, 15], 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 pancreatic cancers [16], but no mutations have been reported in gastric cancers [17]. 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 [18]. 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 [19-22].

DCC receptors are distributed along the length of the epithelium in the intestines, whereas netrin-1 is differentially expressed and forms a gradient within the gastrointestinal tract [21]. 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 is present at the tip of the villi, where many cells are undergoing apoptosis and are 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 [21]. This study 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 showed 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 [21].

Similar to *DCC* receptors, a reduced expression of UNC5A, UNC5B, and *UNC5C* has also been reported in human cancers [23]. In particular, in colorectal cancer a two-fold downregulation of *UNC5C* expression compared with the corresponding normal tissues was observed in approximately 70% of gastric cancer cases. This region is located at 4q21-23, often a target of deletion in gastric cancer, and is associated with epigenetic gene inactivation, such as promoter methylation [24, 25, 23].

In our study we hypothesized that downregulation of *DCC* and *UNC5C* play an important growth-regulatory function in gastric tumorigenesis, which was addressed by investigating a panel of gastric and colorectal 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

As shown in **Table 1,** of 98 gastric cancer patients, 34 patients were female (35%), and 48 tumors were pathologically diagnosed as differentiated (49%). According to the TNM stages, 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 microsatellite instability (MSI; 13%). The mean LOH ratio of the 98 tumors was 0.24 [Standard Distribution (SD), $\pm$ 0.3].

CIN phenotype was categorized by calculating a LOH ratio of the informative markers of the seven polymorphic microsatellite sequences, independently from the *UNC5C* and *DCC* loci. When a tumor showed LOH ratio over 0, the tumor was categorized as CIN-positive. By this criterion, of 98 gastric cancers, 50 tumors (51%) were classified as CIN-positive.

Direct sequencing of gastric cancer specimens determined the proportion of *KRAS*, *BRAF*, and *PIK3CA* mutations (**Table 1**). Mutations in the *KRAS* codon 12 (5%, n = 5/98) and *KRAS* codon 13 (1%, n = 1/98), *BRAF* codon 600 (0%, n = 0/98), *PIK3CA* codon 545 (1%, n = 1/98), and *PIK3CA* codon 1047 (3%, n = 3/98) were detected in gastric cancers. *KRAS* codon 12 mutations consisted of G12D (35G to A, n = 4), G12R (34G to C; n = 1) and codon 13 mutations consisted of G13D (38G to A; n = 1). Interestingly, a tumor displayed both *KRAS* codon 12 and 13 mutations (**Figure 1A**). *PIK3CA* exon 9 mutations comprised E545K (1633G to A, n = 1), and exon 20 mutations comprised H1047R (3140A to G; n = 3). Further, we determined the infection status of *H. pylori* by recovering the *cagA* genotype (**Figure 1B**). Through this analysis, we could recover the *cagA* sequence from 70 gastric cancer tissues (71%).

Methylation Status of DCC in Gastric Cancer Specimens and Association of Clinicopathological Features

We investigated *DCC* methylation status in a cohort of 98 gastric cancers and 105 normal gastric mucosa specimens and analyzed these results as continuous variables (**Figure 2A, B**). We found that 56 of 98 gastric cancers (57%) and 31 of 105 normal

gastric mucosa (29.5%) displayed over 1.0% methylation in the *DCC* promoter. The mean methylation levels were 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 their normal gastric mucosa specimens that displayed over 1.0% methylation ($P < .0001$, Wilcoxon/Kruskal–Wallis test; **Figure 2C**). Therefore, we defined *DCC* methylation at 5% or more as a continuous variable [i.e., ≥5.0% methylation as methylation-positive (methylated) and <5.0% methylation as methylation-negative (unmethylated)]. Using this criterion, we observed *DCC* methylated cases in 44 of 98 gastric cancers (45%) and in 9 of 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 the 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.

LOH of 18q Locus Associates with CIN Phenotype in Gastric Cancer

The frequencies of 18q LOH at each microsatellite marker for *DCC* were 14 of 41 informative cases (24%) at D18S35, 17 of 55 (31%) at D18S69, and 21 of 58 (36%) at D18S58. We categorized 18q LOH-positive as tumors showing LOH in at least one of the three microsatellite markers for 18q LOH. By this criteria, 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 the informative cases, the frequency of gastric cancer with distant metastases was the highest in 18q LOH-positive gastric cancers compared with 18q LOH-negative cancers (25% vs. 9%, $P = 0.041$, **Table 2**). The LOH ratios calculated on the other seven loci was also significantly higher 18q LOH-positive than in 18q LOH-negative tumors (0.44 vs. 0.10, $P < .0001$, **Table 2**). According to these results, when a tumor showed LOH ratio more than 0, the tumor was categorized as CIN-positive. Further, CIN-positive

gastric cancers which were also 18q LOH-positive were significantly more frequent than those which were 18q LOH-negative (74% in 18q LOH-positive vs. 38% in 18q LOH-negative, $P = 0.0017$, **Table 2**).

Reduction of DCC Expression Requires both Genetic and Epigenetic Alterations

Next, we investigated DCC protein expression in 86 gastric cancer tissue samples. Representative examples of immunohistochemistry (IHC) staining results are shown in **Figure 2D–F**. We categorized tumors into following three groups based upon the IHC results analyzed as a categorical variable: complete loss of DCC expression (8 cases, 9%, **Figure 2D**), focal loss of DCC expression (38 cases, 44%, **Figure 2E**), and positive DCC protein expression (40 cases, 47%, **Figure 2F**). Among 86 gastric cancers, 46 cases (53%) displayed reduction of DCC expression. 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 of 7 cancers (71%) demonstrated both *DCC* promoter methylation and 18q LOH. By contrast, 8 of 33 cancers (24%) showed focal loss of DCC expression and both methylation and LOH, 5 of 33 cancers (15%) showed LOH alone, and 12 of 33 cancers (36%) displayed methylation alone. Among the cancers that were positive for DCC expression, only 2 of 34 cancers (6%) demonstrated both methylation and LOH, 13 of 34 (38%) cancers LOH alone, and 8 of 34 cancers (24%) methylation alone (cancers showing both *DCC* methylation and 18q LOH vs. the others, $P = 0.0048$, Pearson chi-square test; **Figure 2G**). Our data suggest that reduction of DCC expression may require dense methylation in the promoter CpGs and LOH of the 18q locus according to the two-hit theory[26].

Association between Clinicopathological Features and Genetic/Epigenetic Alterations of DCC in Gastric Cancer

Since both epigenetic and genetic alterations are critical for DCC suppression, we investigated the relationship between epigenetic and genetic alteration in the *DCC* gene with various clinicopathological features. Of 98 gastric cancers, 15 cancers were categorized as noninformative, 25 cancers were categorized as negative for *DCC*

alterations, and 58 cancers were categorized as positive for *DCC* alterations. Among clinocopahological features, LOH ratio and distribution of CIN phenotype were significantly different between cancers with negative and positive for *DCC* alterations (**Table 2**).

Among the 58 cancers with *DCC* alterations, 17 tumors showed alterations in both *DCC* methylation and 18q LOH, 19 had 18q LOH alone, and 22 cancers harbored *DCC* methylation alone. The LOH ratio calculated by other 7 loci was significantly highest in gastric cancers with both *DCC* methylation and 18q LOH (mean LOH ratio: 0.44 $\pm$ 0.39 SD) and in cancers with 18q LOH alone (mean LOH ratio: 0.44 $\pm$ 0.34 SD), intermediate in cancers with *DCC* methylation alone (mean LOH ratio: 0.16 $\pm$ 0.18 SD), and the lowest in cancers negative for *DCC* alterations (mean LOH ratio: 0.05 $\pm$ 0.10SD, $P < .0001$, Wilcoxon/Kruskal–Wallis test). When we categorized gastric cancers with LOH ratios over 0 as CIN-positive, 12 of 17 gastric cancers (71%) with both *DCC* methylation and 18q LOH, 13 of 17 gastric cancer (76%) with 18q LOH alone, 12 of 22 gastric cancer (55%) with *DCC* methylation alone, and 6 of 25 cancers (24%) negative for *DCC* alterations were categorized as CIN-positive ($P = 0.0025$, chi-square test). Our data suggest that *DCC* alterations caused by both epigenetic and genetic alterations were significantly associated with gastric cancer with CIN phenotype.

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. A panel of representative combined bisulfite restriction analysis (COBRA) results are depicted in **Figure 3A–C**. We analyzed these results by using *UNC5C* methylation levels as continuous variables. We found that 39 of 98 gastric cancer (40%) and 16 of 105 normal gastric mucosa (15%) displayed over 1% methylation in the *UNC5C* promoter. Of the samples that showed over 1% methylation in the *UNC5C* promoter, the mean methylation levels of *UNC5C* was significantly higher in gastric cancers compared with their 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; $P < .0001$, Wilcoxon/Kruskal–Wallis test; **Figure 4C**]. By using these results, we optimized a cutoff value of *UNC5C* methylation at 5% ($\geq$ 5%

methylation as positive and <5% methylation as negative). Using this cutoff value, 31 of 98 gastric cancers (32%) and 5 of 105 normal gastric mucosa (5%) were diagnosed as *UNC5C* methylated. Next, we examined associations between *UNC5C* promoter methylation status and the clinicopathological and genetic features in gastric cancers. *UNC5C* methylation showed a significant association with the 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 the *UNC5C* methylation status and any of the other variables.

Expression and Methylation Status of UNC5C in Gastric Cancer Cell Lines

To assess associations between UNC5C expression status and epigenetic and genetic alterations in the *UNC5C* locus, we performed IHC staining for *UNC5C* protein expression. Unfortunately, we could not analyze the expression of *UNC5C* protein due to lack of adequate antibodies. Therefore, by examining messenger RNA levels by reverse transcription polymerase chain reaction (RT-PCR), we examined associations between *UNC5C* expression and CpG methylation status in the *UNC5C* promoter region in two gastric cancer cell lines (MKN28 and KATO III), three colorectal cancer cell lines (SW48, SW480, and SW837), and one colon fibroblast cell line (CCD18Co). All cell lines, except for CCD18Co cells, lacked expression of the *UNC5C* gene transcripts (**Figure 3B**). Because *UNC5C* expression is proposed to be controlled by the CpGs promoter methylation status, we measured the methylation level of the *UNC5C* promoter by COBRA and observed that all cell lines with loss of *UNC5C* expression were hypermethylated at the *UNC5C* promoter. In contrast, CCD18Co cells, which expressed *UNC5C*, did not show *UNC5C* promoter methylation, as described previously (**Figure 4D**).

LOH of 4q Locus Associates 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 D4S2381, 23% (10/44) at D4S470, and 28% (12/43) at D4S1559. We defined 4q LOH-positive tumors 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 vs. 39% in 4q LOH-negative, $P = 0.036$).

Similar to 18q LOH, we found a strong correlation between 4q LOH and CIN phenotype. The LOH ratio calculated on other 7 loci was significantly higher in gastric cancers with 4q LOH compared with those without 4q LOH (0.40 vs. 0.15, $P < .0001$, **Table 3**). According to these results, when a tumor showed LOH ratio over 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 vs. 37% in 4q LOH-negative, $P = 0.0003$, **Table 3**). Whereas 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 nonsignificant 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 noninformative, 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 (stage I and II vs stage III and IV, $P = 0.02$, Fisher's exact test) and in advanced categories for lymph node metastasis (N0 and N1 vs N2 and N3, $P = 0.02$, Fisher's exact test).

Among the 40 cancers with *UNC5C* alterations, 10 cancers showed alterations in both *UNC5C* methylation and 4q LOH, 13 cancers showed 4q LOH alone, and 17 cancers showed *UNC5C* methylation alone. The LOH ratio calculated on the other 7 loci was significantly higher in cancers with both *UNC5C* methylation and 4q LOH (mean LOH ratio: 0.45 ± 0.36 SD) and in those with 4q LOH alone (0.37 ± 0.25 SD), intermediate in cancers with *UNC5C* methylation alone (0.24 ± 0.29 SD), and lowest in cancers negative for *UNC5C* alterations (0.11 ± 0.22 SD, $P = 0.0002$, Wilcoxon/Kruskal–Wallis test). When we categorized the cancers that showed LOH ratios over 0 as CIN-positive, 8 of 10 gastric cancer (80%) with both *UNC5C* methylation and 4q LOH, 11 of 13 gastric cancer (85%) with 4q LOH alone, 9 of 17

gastric cancers (53%) with *UNC5C* methylation alone, and 11 of 39 cancers (30%) negative for *UNC5C* alterations were categorized as CIN-positive ($P = 0.001$, chi-square test). Similarly 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 Losses of Netrin-1 Receptors Occurs According to 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 (**Figure 4 and Supplementary Table 1**). As shown in **Figure 4A**, concurrent alterations in the *DCC* and *UNC5C* genes were observed significantly more commonly in advanced stages [61% (14/ 23) and 70% (7/10) for stage III and IV, respectively] than in earlier-stage cancers [9% (1/11) and 32% (7/22) for stage I and II, respectively; $P = 0.007$]. By segregating gastric cancers based on individual defects in either *UNC5C* or *DCC* and their relationship with tumor stage, we found that *UNC5C* alterations gradually increased according to the progression of the TNM stage (**Table 3**), whereas *DCC* alterations were constantly observed with high frequency in all TNM stages (**Table 2**). Hence, such differential feature found in each gene indicates that cumulative alterations of netrin-1 receptors are associated with gastric cancer progression. With respect to the factors that determine the TNM classification, cumulative loss of netrin-1 receptors was more strongly associated with the degree of regional lymph node metastasis (N factor, **Figure 4C**) compared with the tumor status of T factors (**Figure 4B**). Interestingly, gastric cancers with defects in either *UNC5C* or *DCC* did not show distant metastasis (**Figure 4D)**, suggesting that the cumulative alterations of netrin-1 receptors was a late event in gastric cancer progression, and was significantly associated with CIN-positive gastric cancers through increasing the LOH ratio (**Figure 4G, H**) rather than MSI and mutational status ((**Figure 4E, F**). On the other hand, there were no

significant associations between cumulative loss of netrin-1 receptors and any other clinicopathological variables **(Supplementary Figure**).

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 tissues. We found frequency of gastric cancers with concurrent alterations in the *DCC* and *UNC5C* genes increased in a stage-dependent manner. Segregating 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 high frequency: 10 of 16 (63%) in stage I, 19 of 25 (76%) in stage II, 19 of 29 (66%) in stage III, and 10 of 13 (77%) in stage IV (**Table 2**); whereas, *UNC5C* alterations gradually increased according to the progression of the TNM stage: 2 of 13 (15%) in stage I, 12 of 25 (48%) in stage II, 19 of 30 (63%) in stage III, and 7 of 11 (64%) in stage IV (**Table 3**). Both *DCC* and *UNC5C* were inactivated in 97% of CIN-positive gastric cancers and in 55% of CIN-negative gastric cancers and that 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 the tumor stages, whereas *UNC5C* inactivation accrues gradually during multistep gastric carcinogenesis.

The netrin-1 receptor, *DCC*, was discovered as a putative tumor-suppressor gene in colorectal cancer[18]. *DCC* is located on chromosome 18q, which is the most common deleted chromosomal region in colorectal cancer as well as gastric cancer [11, 27, 10, 28, 29, 12]. The tumor-suppressor role for *DCC* has been questioned in studies that failed to show a clear malignant phenotype in *DCC* knockout mice models [30]. However, more recent studies have challenged this hypothesis and have suggested a role for *DCC* in suppressing tumor growth and metastasis [21, 22]. Recent indications that *DCC* serves as a dependence receptor for netrin-1 have renewed the notion that *DCC* may function as a proapoptotic growth suppressor when not bound to its ligand [31, 32, 29, 33]. In the gastrointestinal tract, netrin-1 performs 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 [21, 31, 32]. In line

with previous studies [34, 35], in this study we demonstrated that methylation-induced silencing of *DCC* as well as allelic loss of 18q was critical for loss of *DCC* expression. Thus, reduction of *DCC* expression might require dense CpGs promoter methylation and LOH of the 18q locus according to the two-hit theory[26].

The netrin-1 receptor, *UNC5C*, was also discovered as a putative tumor-suppressor gene in various tumors, including gastric cancer [23, 24]. It was suggested that loss of *UNC5C* was caused by allelic losses of chromosome 4q, and mutations were rarely observed [23]. Allelic losses at the 4q locus have been reported previously in several human cancers, and the frequencies ranged from 23%–39% [23, 36, 37]. 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 D4S2381, 23% (10/44) on D4S470, and 28% (12/43) on D4S1559. Finally, gastric cancers demonstrating allelic loss at 4q were found in 29% (23 of 79) of the informative cases. Another mechanism causing 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 [25]. As is the case in colorectal cancer, our results demonstrated that two gastric cancer cell lines (MKN28 and KATO III) lacking *UNC5C* expression showed dense methylation in the *UNC5C* promoter. In clinical specimens, 31 of 98 gastric cancers (32%) and 5 of 105 normal gastric mucosa tissues (5%) harbored *UNC5C* methylation. Therefore, next 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 determinants for lack of DCC. However, we could not perform UNC5C IHC on the clinical materials because of lack of an adequate antibody for tissue staining.

Because both *DCC* and *UNC5C* share the same netrin ligand and are colocalized in the gut [21, 20, 23, 38], 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 evolution 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.

The presence of *H. Pylori* in human gastric mucosa is a well-known inducer of chronic inflammation and gastric cancers and associated with high incidences of aberrant DNA methylation [39-42]. So we detected *H. Pylori* infection by recovering the *cagA* repeat sequence from gastric cancer tissues as well as normal gastric mucosa. Of 98 cancers, 70 cancers could recovered the cagA sequence. However, there was no association between presences of the cagA sequence in cancer tissues and their clinicopahological and molecular features, especially *DCC* nor *UNC5C* methylation incidences. Conversely, with respect to normal counterpart gastric mucosa, total of 102 normal gastric mucosa was available for analyzing the presence of *cagA* sequence in this study. Of 102 gastric mucosa, 79 (74%) gastric mucosa successfully recovered the *cagA* sequence. Interestingly when we defined *DCC* and *UNC5C* methylation at 1% or more as a continuous variable [i.e., $\geq$1.0% methylation as methylation-positive (methylated) and <1.0% methylation as methylation-negative (unmethylated)], only methylation of *UNC5C* was significantly associated with the presence of *cagA* sequence in normal gastric mucosa (data not shown), suggesting that inflammatory processes associated with *H. Pylori* infection causes aberrant methylation in the *UNC5C* promoter CpGs but not in the *DCC* promoter. Thus, our result suggested that *H. Pylori* infection will not induce aberrant methylation randomly, but in target loci by some certain signal cascade. To address questions underling *H. Pylori* infection and epigenetic changes, further investigation will required.

Conclusions

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

Methods

Primary Gastric Tissues

We collected tissue specimens of primary gastric cancer and their matched normal gastric mucosa from 105 patients who had undergone surgery at the Okayama University Hospital (Okayama, Japan). Of 105 gastric cancer patients, 7 cancer cases did not have sufficient tumor tissue for analysis. Thus, in this study, a total of 98 gastric cancer tissues and 105 matched normal gastric mucosa tissues were analyzed. All normal gastric mucosa tissues 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 ethical committee of the Okayama University Hospital. All patients also gave informed consent for usage of their data for future analyses. The histological diagnosis was made 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 (7th edition).

Cell Lines

Two human gastric cancer cell lines (MKN28 and KATO III), three human colorectal cancer cell lines (SW48, SW480, and SW837), and one human colon fibroblast cell line (CCD18Co) were purchased from American Type Culture Collections (Manassas, VA, 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% CO_2.

DNA and RNA Extraction

Genomic DNA from the cell lines was extracted using QIAamp DNA mini kits (Qiagen, Valencia, CA, USA). All gastric cancers and normal gastric mucosa 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 cultured cell lines was obtained using the TRIzol reagent (Invitrogen Life Technologies Inc, Carlsbad, CA, USA).

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, Carlsbad, CA, USA) with a total of 1.0 μg RNA. RT-PCR was performed using *UNC5C* and *β-actin* specific primer pairs (**Supplementary Table 2**). The PCR products were electrophoresed on a 3.5% agarose gel.

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 *UNC5C* promoters in gastric tissues and cell lines was analyzed by combined bisulfite restriction analysis (COBRA, **Supplementary Table 2**). COBRA was carried out in a 24.0-μL PCR reaction containing 12.0 μL of HotStarTaq Master Mix kit (Qiagen, Valencia, CA, USA) and 0.4 μM of each primer. The PCR products were digested with a restriction enzyme *Hha*I (New England Biolabs Inc, Ipswich, MA, USA) at 37^{o}C overnight. The PCR products were electrophoresed on a 3.0% agarose gel. The percentages of methylated *Hha*I sites were calculated by determining the ratios between the *Hha*I-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 each gene was used to determine LOH at chromosomes 18q21 for *DCC* and 4q21-23 for *UNC5C* (**Supplementary Table 2**). PCR amplifications were performed on genomic DNA templates from both tumor and normal mucosa tissues using fluorescently labeled primers. The amplified 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 of at least 40% of the signal intensity was considered indicative of LOH.

In addition, to examine the association between disorders of netrin-1 receptors and CIN phenotype, we analyzed additional 7 sets of polymorphic microsatellite sequences that are tightly linked to known tumor suppressor genes and DNA MMR genes, including the *MYCL* locus on 1p34 (MYCL), the h*MSH2* locus on 2p16 (D2S123), the *APC* locus on 5q21 (D5S346, D5S107), the UNC5D locus on 8p12 (D8S87), and the *p53* locus on 17p13 (D17S250, TP53)[43]. Of 98 gastric cancer patients, 96 patients displayed at least one marker informative for the LOH status. Since 2 patients showed noninformative LOH at all seven microsatellite sequences, the further analyses were done on the 96 informative cases. CIN phenotype was categorized by calculating a LOH ratio of the informative markers of the 7 polymorphic microsatellite sequences, independently from *UNC5C* and *DCC* loci. When a tumor showed a LOH ratio over 0, the tumor was categorized as CIN-positive.

MSI Analysis and Definition of MSI Phenotype

The MSI status was analyzed in all 98 gastric cancer patients by using 3 mononucleotide repeat markers (BAT26, NR21, and NR27) as described previously[44]. When at least 1 or more mononucleotide repeat markers displayed microsatellite instability, tumors were defined to have an MSI phenotype and the 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 were analyzed in all 98 patients as described previously [45]. In addition, *PIK3CA* exon 9 and 20 mutation status were also analyzed by direct sequencing. PCR and sequencing were performed using *PIK3CA* exon 9 and 20 specific primer pairs (**Supplementary Table 2**). The amplified PCR products were electrophoresed on an ABI 310R Genetic analyzer (Applied Biosystems, Foster City, CA, USA).

Detection of H. Pylori

To determine *H. pylori* infection status, we recovered the 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 amplifications performed on genomic DNA templates from tumor tissues. We modified the primer design to make PCR products shorter than the PCR products described previously [46, 47]. PCR was carried out in a 24-μL PCR reaction containing 12 μL of HotStarTaq Master Mix kit (Qiagen, Valencia, CA, USA) and 0.4 μM of each primer (**Supplementary Table 2**). 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 the citrate buffer (pH 6.0), endogenous peroxidase was blocked with 3% H_2O_2. 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 positively staining admixed or surrounding nonneoplastic 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 *UNC5C* methylation levels were analyzed as continuous variables, as were computed means, standard error of the means, and

standard distributions. Next, the methylation status of the *DCC* and *UNC5C* promoter was analyzed as a categorical variable (positive, methylation level $\geq$ 5%; negative, methylation level < 5%). Differences in frequencies were evaluated by the Fisher' exact test, the chi-square test, or the Wilcoxon/Kruskal–Wallis test. Whenever the Kruskal–Wallis test indicated differences among these subgroups, further pair-wise comparisons for each of the subgroup was performed by a nonparametric multiple comparison method using the Steel–Dwass test. All reported *P* values were two-sided and a *P* value of less than .05 was considered statistically significant.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

KT performed methylation and LOH analyses of both genes in the clinical materials, 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 clinicopathological data. TT performed the IHC and pathological findings. NK and YT assisted with methylation and LOH analyses of the both genes of clinical materials. HT and DSS assisted with the methylation and LOH analyses in the cell lines and partially in the clinical materials. NN assisted with the cell line and IHC studies. AG assisted with the interpretation of the data, co-designed the project, and revised the manuscript. TF provided patients' samples and clinicopathological data, assisted with the interpretation of the data, and revised the manuscript. All authors read and approved the final manuscript.

Acknowledgements

The authors would like to thank Mr. Toru Nakai, Mrs. Take Yamanishi, and Mr. Akihiro Nyuuya for technical assistance and Enago (www.enago.jp) for the English language editing. This work was supported by KAKENHI (19390351 and 20590572)

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Figures

Figure 1- 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 determine *H. pylori* infection, EPIYA repeat sequences in the *cagA* protein were recovered from clinical materials. We found two type of EPIYA repeat sequences, one is 159 bps and another is 261 bps. SM denotes a size marker; P and N denote positivity and negativity of EPIYA- repeat sequences, respectively.

Figure 2- *DCC* promoter methylation and immunohistochemistry analyses

(A) Schematic representation of *DCC* gene promoter regions analyzed by combined bisulfite restriction analysis (COBRA). Gray and black squares represent the untranslated and the coding exon 1 region, respectively; arrows on the squares indicate transcriptional start sites; vertical lines indicate CpG sites; white diamonds represent the restriction sites for HhaI; thick horizontal lines depict the locations of COBRA products; arrows on the thick horizontal lines denote COBRA primers. (B) Representative results of COBRA of *DCC.* Arrows indicate methylated alleles; M denotes methylation; U denotes unmethylation; Mc denotes the methylated control. SM denotes a size marker. (C) Results of *DCC* methylation as continuous variables. In the box plot diagrams, the horizontal line within each box represents the median; the limits of each box are the interquartile ranges, the whiskers are the maximum and minimum values. Immunohistochemistry analysis for *DCC* (D–F). Nuclei of tumor cells are completely negatively (D), focally negatively (E) and positively (F) stained. (G) Association between epigenetic/genetic alteration and *DCC* expression.

Figure 3- *UNC5C* promoter methylation analysis

(A) Schematic representation of *UNC5C* gene promoter regions analyzed by COBRA. Gray and black squares represent the untranslated and the coding exon 1 region, respectively; arrows on the squares indicate transcriptional start sites; vertical lines indicate CpG sites; white diamonds represent the restriction sites for HhaI; thick horizontal lines depict the locations of COBRA products; arrows on the thick horizontal

lines denote COBRA primers. (B) Representative results of COBRA of *UNC5C*. Arrows indicate methylated alleles; M denotes methylation; U denotes unmethylation; Mc denotes methylated control; SM denotes a size marker. (C) Results of *DCC* methylation as continuous variables. In the box plot diagrams, the horizontal line within each box represents the median; the limits of each box are the interquartile ranges, the whiskers are the maximum and minimum values. (D) Correlation of *UNC5C* methylation and loss of *UNC5C* mRNA expression in gastric cancer and colorectal cancer cell lines. Two gastric cancer cell lines, three colorectal cancer cell lines, and CCD18Co cells were analyzed for mRNA expression by RT-PCR of *UNC5C* and *beta-actin* genes. The lowest panel illustrates the methylation profile obtained from COBRA.

Figure 4- Association between alteration patterns in netrin-1 receptors and clinicopathological features in gastric cancers

Correlation between alterations in netrin-1 receptors and TNM stage (A), depths of invasion (B), degree of regional lymph node metastasis (C), presence of distant metastasis (D), MSI status (E), *KRAS/BRAF/PIC3CA* mutation status (F), and CIN phenotype. * Two cases could not be evaluated for CIN phenotype. (H) Association between alteration patterns in netrin-1 receptors and LOH ratio. In the box plot diagrams, the horizontal line within each box represents the median; the limits of each box are the interquartile ranges, the whiskers are the maximum and minimum values. Asterisks and the numbers denote the mean value of LOH ratio.

Tables

Table 1 - Characteristics of gastric cancer patients

Table 2 - Association between epigenetic/genetic alterations of the *DCC* gene and clinicopathological features in gastric cancers

Table 3 - Association between epigenetic/genetic alterations of the *UNC5C* gene and clinicopathological features in gastric cancers

Additional files

Additional file 1- Supplementary Figure- Association between alteration patterns in netrin-1 receptors and clinicopathological features in gastric cancers
Correlation between alterations in netrin-1 receptors and gender (A), Histology (B), *H. pylori cagA* expression status (C), and Age. In the box plot diagrams, the horizontal line within each box represents the median, the limits of each box are the interquartile ranges, the whiskers are the maximum and minimum values. Asterisks and the numbers denote the mean value of age at surgery.

Additional file 2 – Supplementary Table 1- Association between alterations in netrin-1 receptors and clinicopathological features in gastric cancers.
Additional file 3 – Supplementary Table 2- Primer sequences

Figure1

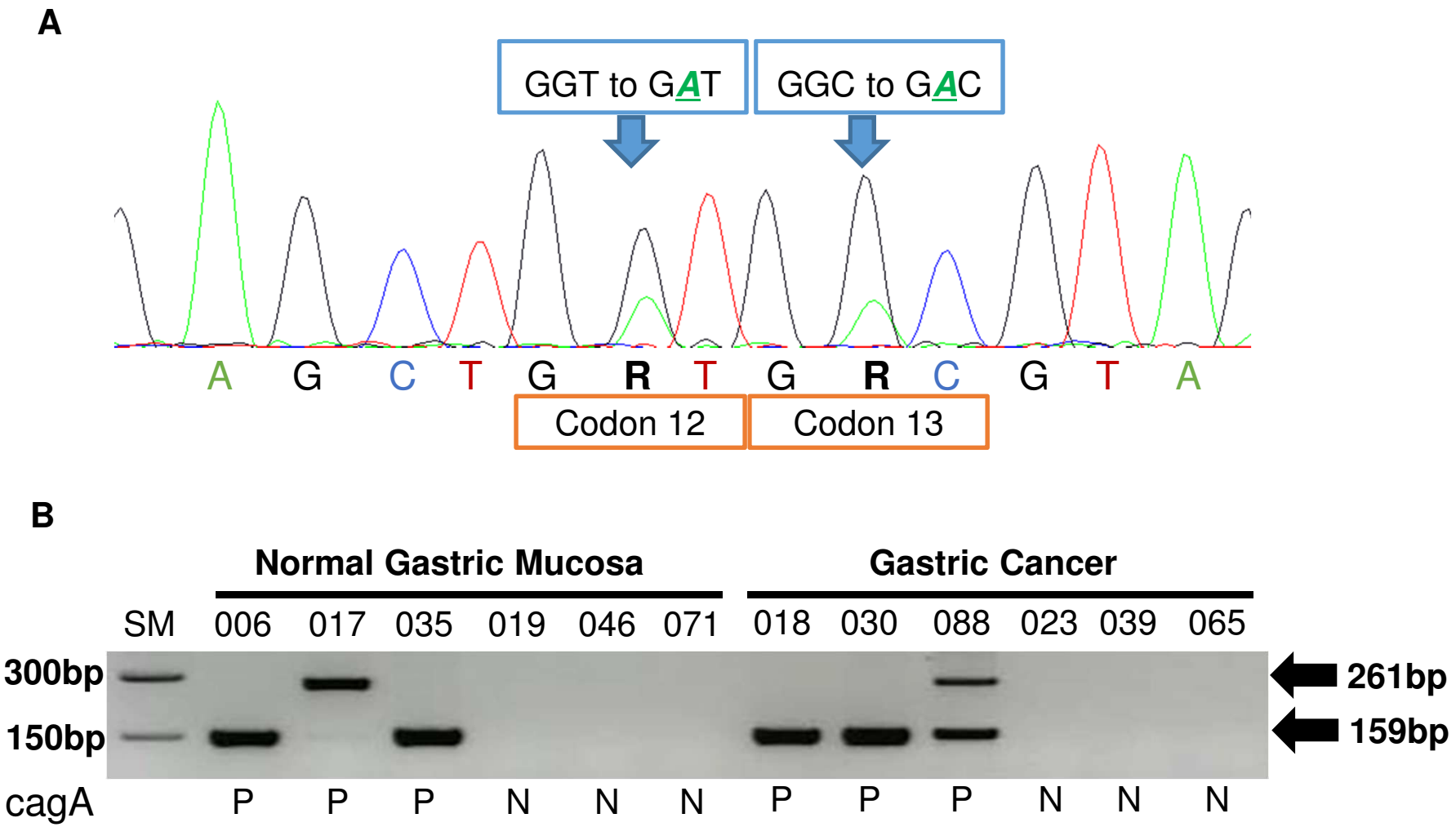

A
GGT to GAT
GGC to GAC
A G C T G R T G R C G T A
Codon 12
Codon 13
B
Normal Gastric Mucosa
Gastric Cancer
SM 006 017 035 019 046 071 018 030 088 023 039 065
300bp
150bp
261bp
159bp
cagA P P P N N N P P P N N N

Figure 2

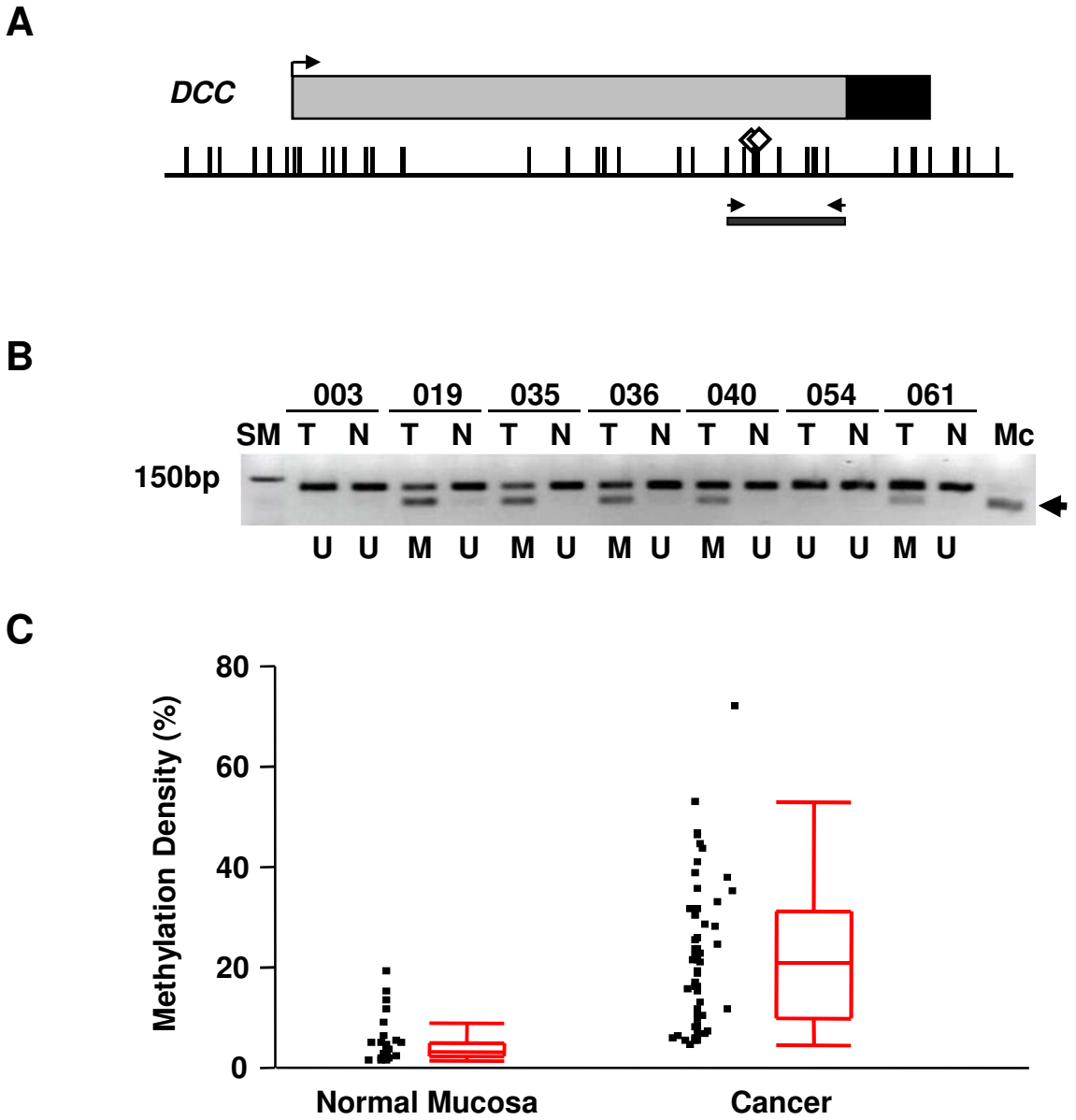
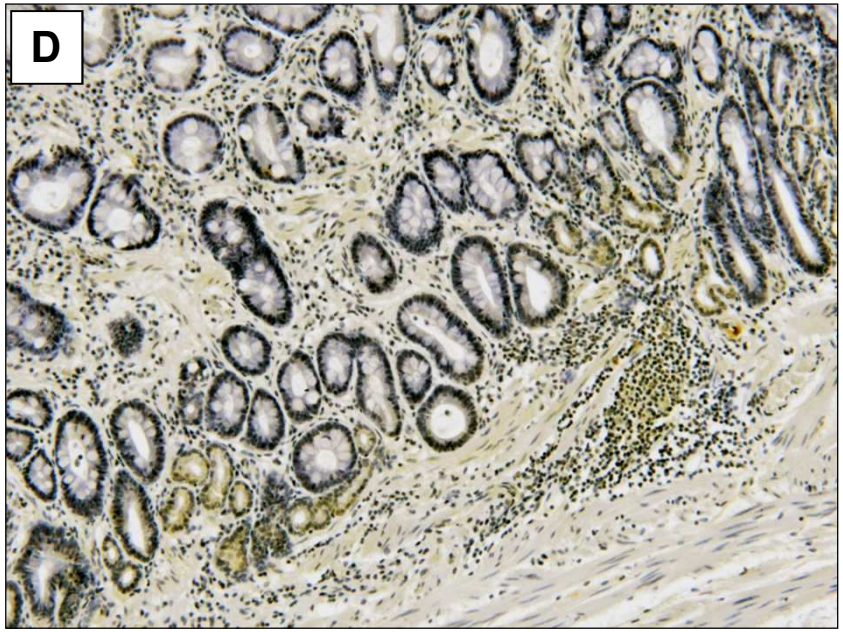
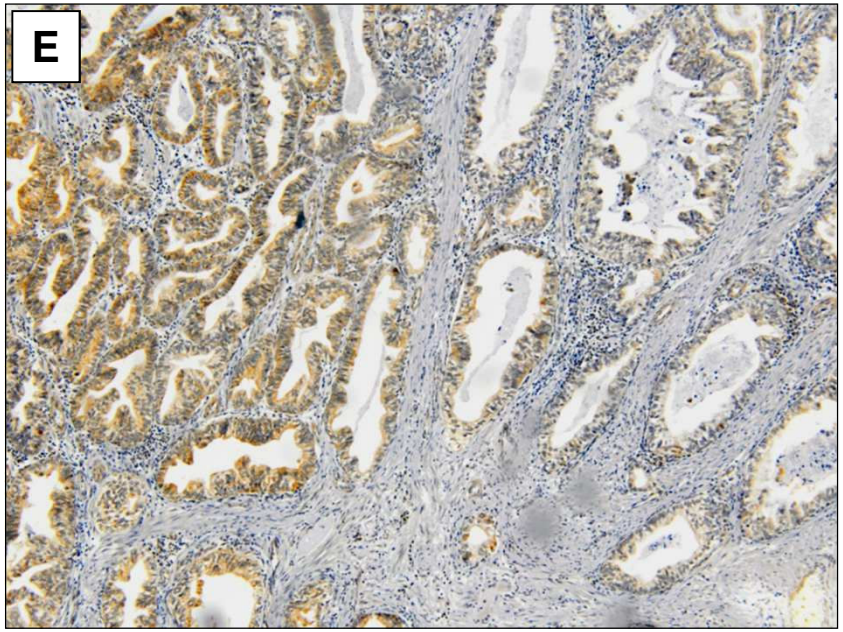
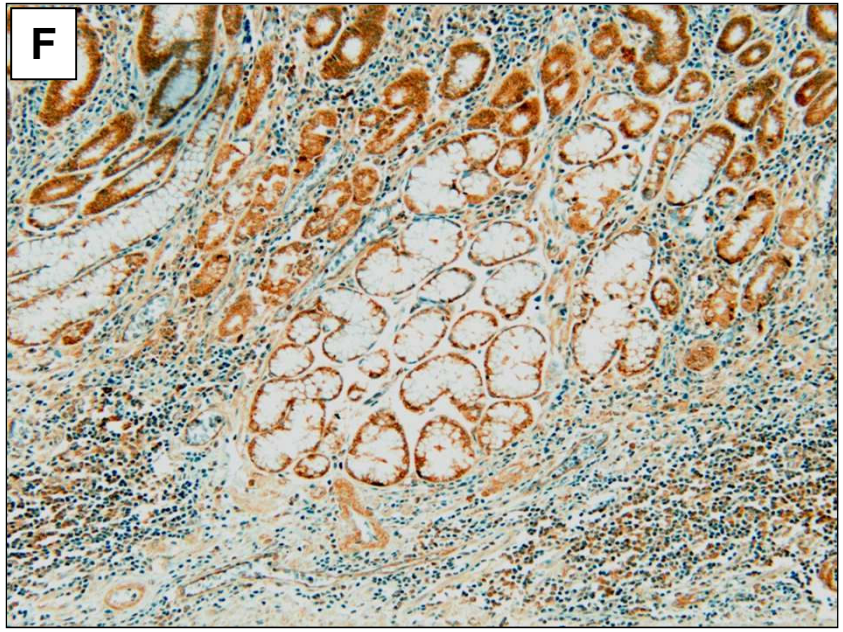

A
DCC
B
003
019
035
036
040
054
061
SM T N T N T N T N T N T N T N Mc
150bp
U U M U M U M U M U U U M U
C
Methylation Density (%)
80
60
40
20
0
Normal Mucosa
Cancer

Figure 2


D


E


F

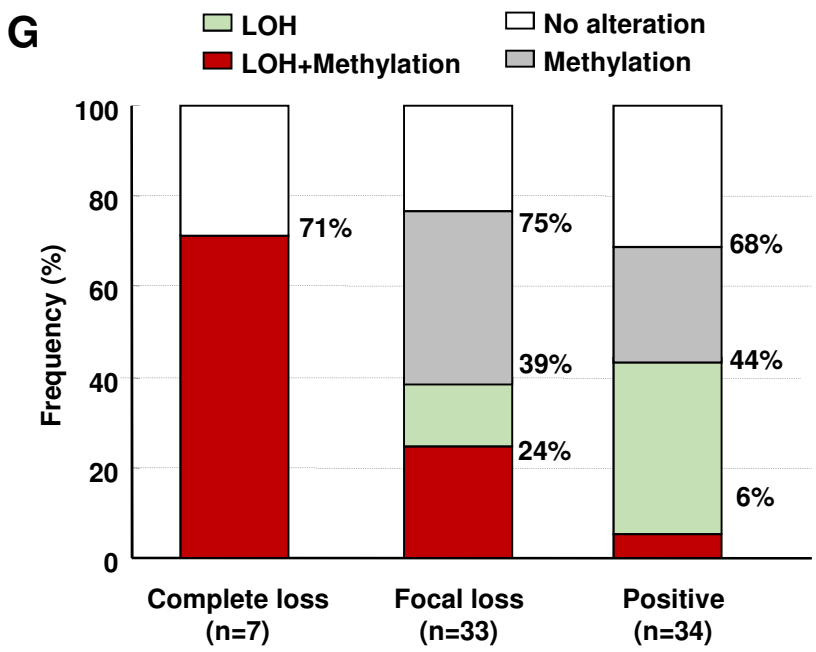

G
LOH
LOH+Methylation
No alteration
Methylation
Frequency (%)
100
80
60
40
20
0
71%
75%
39%
24%
68%
44%
6%
Complete loss
(n=7)
Focal loss
(n=33)
Positive
(n=34)

Figure 3

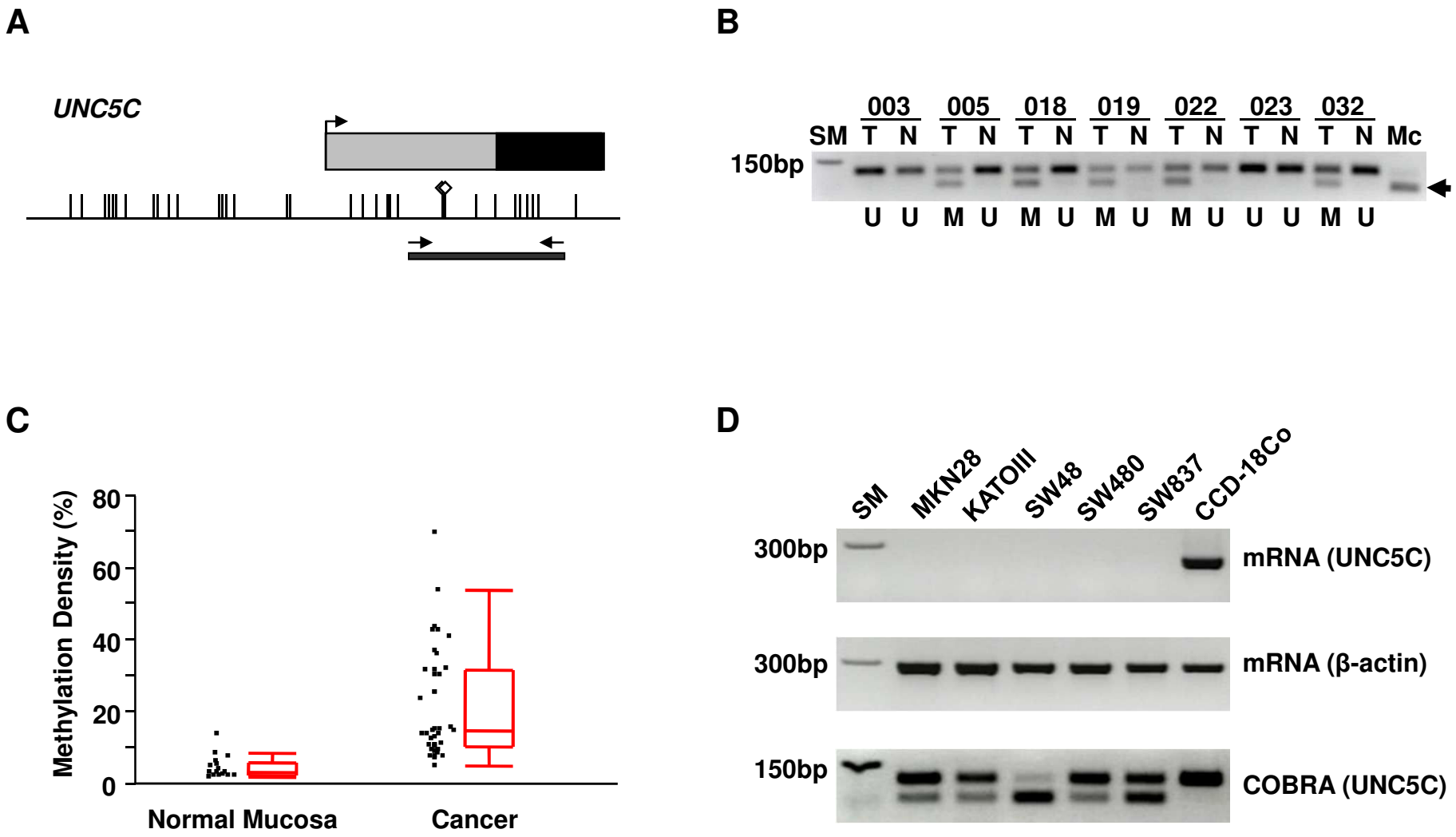

A
UNC5C
B
003
005
018
019
022
023
032
SM T N T N T N T N T N T N T N Mc
150bp
U U M U M U M U M U U U M U
C
Methylation Density (%)
80
60
40
20
0
Normal Mucosa
Cancer
D
SM
MKN28
KATOIII
SW48
SW480
SW837
CCD-18Co
300bp
mRNA (UNC5C)
300bp
mRNA (β-actin)
150bp
COBRA (UNC5C)

Figure 5

Figure 4

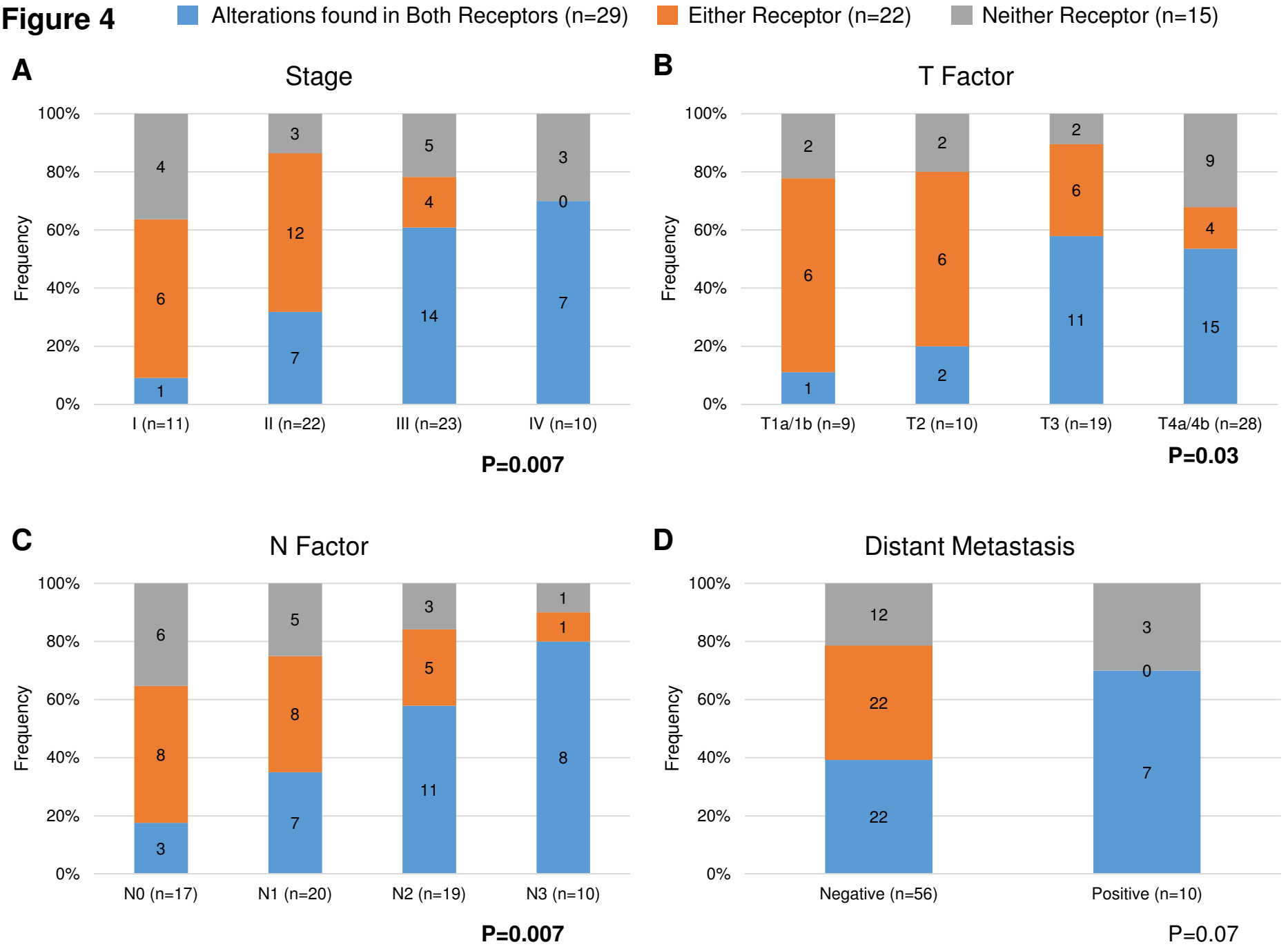


Figure 6

Figure 4

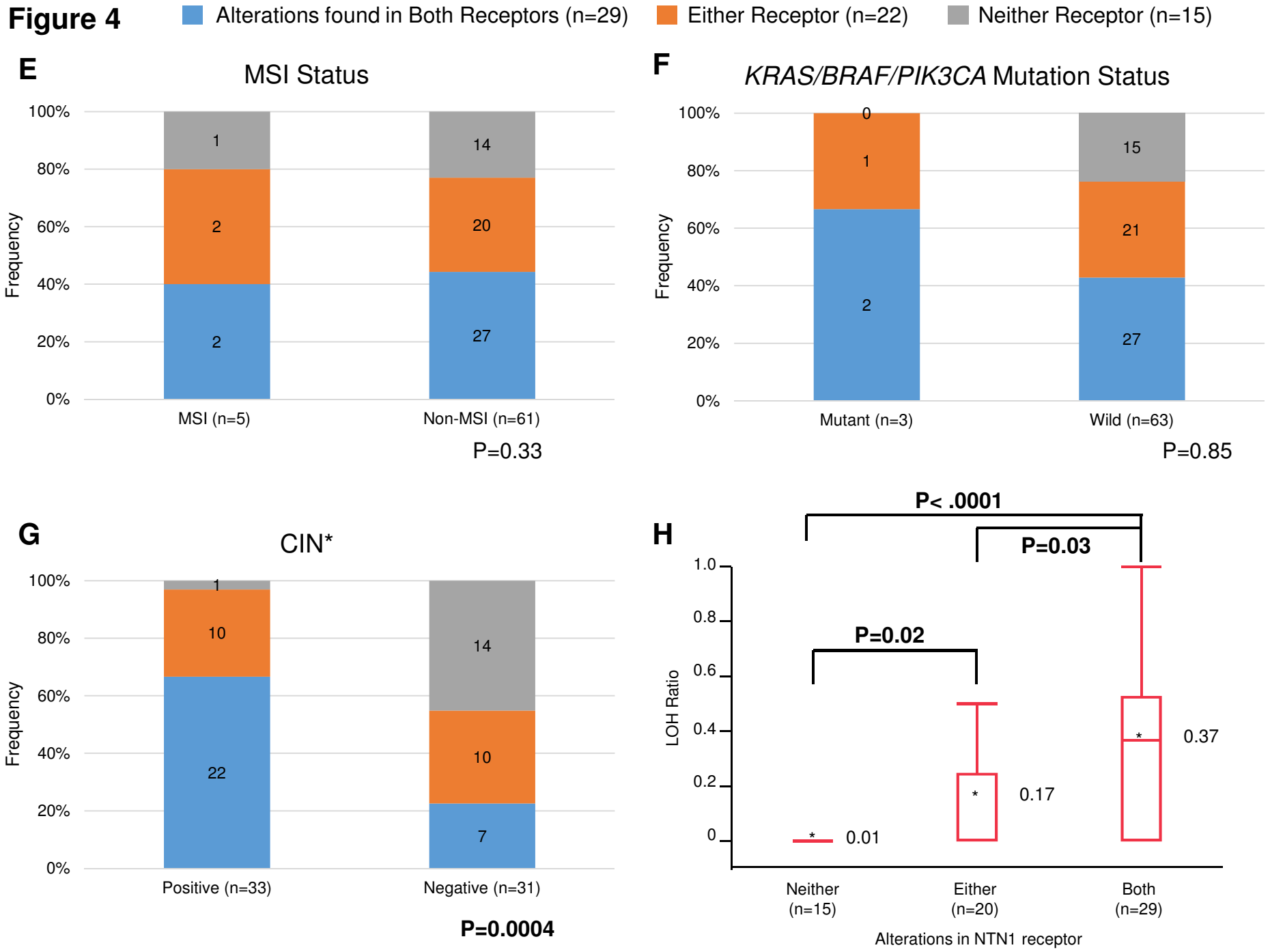
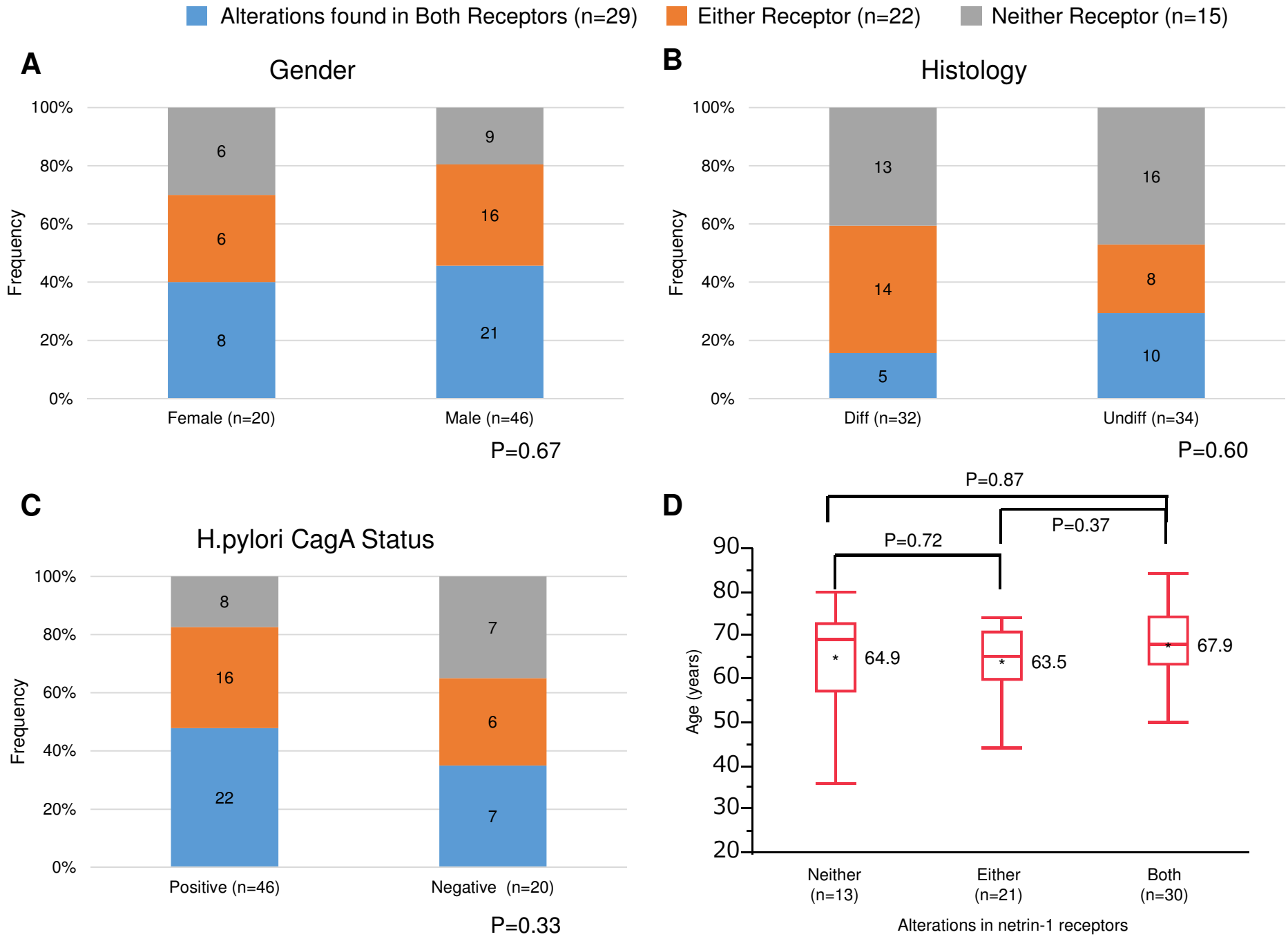


Figure 7

Supplementary Figure 1



Additional files provided with this submission:

Additional file 1: Table1 Toda K et al.xlsx, 10K
http://www.clinicalepigeneticsjournal.com/imedia/6022335301612014/supp1.xlsx
Additional file 2: Table2 Toda K et al.xlsx, 15K
http://www.clinicalepigeneticsjournal.com/imedia/1218217462161201/supp2.xlsx
Additional file 3: Table3 Toda K et al.xlsx, 15K
http://www.clinicalepigeneticsjournal.com/imedia/3593175721612014/supp3.xlsx
Additional file 4: Supplementary Table 1 Toda K et al.xlsx, 11K
http://www.clinicalepigeneticsjournal.com/imedia/1657644607161201/supp4.xlsx
Additional file 5: Supplementary Table 2 Toda K et al.xlsx, 11K
http://www.clinicalepigeneticsjournal.com/imedia/6387191451612014/supp5.xlsx