Dysregulation of Netrin-1 receptors in human colon cancer

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Dysregulation of Netrin-1 receptors in human colon cancer

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The Doctoral Dissertation submitted to the
Department of Medicine,
the Graduate School of Yonsei University
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy

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JUNE 2016

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JUNE 2016

ACKNOWLEDGEMENTS

First and foremost, I would like to give my sincere gratitude to my supervisor Prof. Tae Il Kim for the enthusiastic support of my Ph.D thesis, patience, and generosity. Without his dedicated assistance, this thesis would have never been accomplished. I could not have imagined having a better advisor for my Ph.D study. Besides my supervisor, I would like to appreciate from the bottom of my heart, the rest of my thesis committee: Prof. Won Ho Kim, Prof. Hoguen Kim, Prof. Woo Jin Hyung, and Prof. Dong Soo Han, for their insightful comments and encouragement, but also for the hard question which incented me to widen my research from various perspectives. My sincere thanks also goes to Dr. Boland, and Dr. Goel, who provided me an opportunity to join their team, and who gave access to the laboratory and research facilities. Without their precious support it would not be possible to conduct this research.

Last but not the least, none of this could have happened to me without the love and patience of my family. To parents, you have been always my Champions and will be forever. To my wife and two sons – it would be an understatement to just say that, as a family, we have experienced some ups and downs in the past several years. Every time I was ready to surrender to the odds, you did not let me and I owe you a “really big one” which I cannot pay back enough even for the rest of my life. This dissertation stands as a testament to your unconditional love and encouragement.

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ABSTRACT

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Netrin-1 is a laminin-related secreted protein that performs an important role in intestinal epithelial biology. The netrin-1 pathway may be involved in human tumorigenesis through its interactions with two dependence receptors: deleted in colorectal cancer (*DCC*) and *UNC5C*. These 2 receptors induce apoptosis in the absence of netrin-1, but promote cell survival in its presence. Colorectal Cancers (CRCs) frequently lose *DCC* due to deletions in chromosome 18q, and recent reports indicate that CRCs also exhibit reduced or loss of *UNC5C* expression. The mechanism for the loss of *UNC5C* in CRCs is not clearly understood, since mutations have rarely been observed, and allelic losses have been inconsistently reported. Since *DCC* and *UNC5C* share the same ligand, and both receptors are frequently down regulated in CRC, a better understanding of underlying molecular processes is critical for appreciating the role of netrin pathway in the colonic tumorigenesis. The hypothesis of this thesis was that *UNC5C* is epigenetically silenced in CRC, and that there are interactions between losses of *UNC5C* and *DCC* in colorectal tumorigenesis.

Gene expression and epigenetic analysis of *UNC5C* was examined in a panel of 8 CRC cell lines, 147 primary sporadic CRCs with corresponding normal mucosa (CNM), and 52 colorectal adenomatous polyps (APs). Allelic imbalances at *DCC* were determined in CRCs using markers mapping chromosome 18q. The molecular analyses were compared with genetic and clinico-pathological features.

All 8 CRC cell lines demonstrated *UNC5C* methylation and an associated loss of gene expression. Treatment with a demethylating agent resulted in restoration of gene transcription. *UNC5C* methylation was significantly more frequent in CRCs (76.2%) and APs (63.5%) than in CNM (6%; $p<0.001$). Allelic imbalance at *DCC* was observed in 61% of CRCs. Overall, 89.3% of CRCs had alterations of either netrin receptor. Finally, *UNC5C* methylation occurred predominantly in the earlier lesions (APs and early stage CRC), whereas *DCC* losses were observed more often in advanced CRCs.

I provide first evidence that majority of CRCs harbor defects in netrin-1 receptors, emphasizing the importance of this growth regulatory pathway in cancer. My data also suggest that the timing of the molecular alterations in the two netrin-1 receptors is not random, since *UNC5C* inactivation occurs early, whereas LOH of *DCC* occur in later stages of multistep colorectal carcinogenesis.

Key words: *UNC5C*, *DCC*, colorectal cancer, promoter methylation, loss of heterozygosity, dependence receptor

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I. INTRODUCTION

CRC occurs as a consequence of the successive accumulation of genetic and epigenetic alterations in multiple genes that control epithelial cell growth and other cellular behaviors. Three mechanisms involved in increasing the diversity of gene expression in CRC include: microsatellite instability (MSI), chromosomal instability (CIN) and the CpG island methylator phenotype (CIMP) [1-5]. CIN can be found in approximately half of all sporadic CRCs and is characterized by aneuploidy and large scale chromosomal duplications, deletions, and rearrangements [1]. The discovery of CIMP has been more recent, and represents excessive, aberrant methylation of CpG dinucleotides (or CpG islands) that preferentially occur in the promoter regions of ~50% of tumor suppressor genes [3, 6, 7]. Methylation of these CpG islands and associated modifications in histone complexes can result in transcriptional silencing of genes in an epigenetic manner [3, 8]. MSI, which occurs in 12-15% of sporadic CRCs, is most often a consequence of

CIMP when the DNA mismatch repair gene *hMLH1* becomes a target of epigenetic silencing [9]. Even though some overlap exists between genetic and epigenetic instability pathways in the colon, recent work suggests that CIMP and CIN represent two inversely related mechanisms of genomic instability in CRC [2].

A common feature of CIN in CRC is that these neoplasms frequently demonstrate loss of heterozygosity (LOH) at chromosomes 5q, 17p and 18q, which has been linked to the loss of function of *APC*, *p53* and the *DCC*/*SMAD* genes respectively [1, 10]. Finding frequent losses at 18q was instrumental in the initial discovery of *DCC* [11]. The role of *DCC* as a tumor suppressor gene was questioned because mutations in *DCC* were rarely detected, and mouse studies failed to provide a convincing phenotype in knockout models [12, 13]. The presence of the closely linked *SMAD* genes raised the possibility that the linkage between cancer and LOH at 18q was more directly related to losses of the *SMAD* genes than *DCC* [14]. However, recent studies with new data argue in favor of a role for *DCC* in regulating growth and suppressing metastasis [15-17].

The recent discovery that both *DCC* and *UNC5H* serve as 'dependence receptors' for Netrin-1 (NTN1) have reinvigorated support for a tumor suppressor role of *DCC* in human cancer [15, 16, 18, 19]. NTN1 belongs to a family of laminin-related secreted proteins in the brain and other tissues [20]. It has been suggested that *NTN1* acts through *DCC* and *UNC5H,* which function as dependence receptors in colonic and other tissues [18]. These receptors induce apoptosis when not engaged with their ligand netrin, but mediate signals for proliferation, differentiation or migration when ligand-bound [15, 21, 22]. In the gut, NTN1 plays a role in mucosal integrity, epithelial cell migration, and tissue renewal by inducing cell survival in proliferating crypt progenitors, where netrin levels are high. At the villus tips, where netrin levels are low, both *DCC* and *UNC5H* promote apoptosis and cell shedding, because the death receptors are constitutively expressed throughout the crypt-villus axis [15, 23, 24].

Interestingly, NTN1 is also a ligand for the *UNC5H* receptor family [25, 26]. *UNC5A*, *UNC5B*, *UNC5C* and *UNC5D* are type I transmembrane receptors involved in axonal guidance in the development of the nervous system. Although *UNC5H* receptors are usually expressed in adult human tissues, a recent report highlighted that these receptors are frequently down-regulated in several types of human cancers [27]. The loss of *UNC5C* expression is particularly prominent in CRC [27]. Unlike *DCC* deletions, the molecular mechanisms responsible for the loss of *UNC5C* expression are poorly understood. The only report addressing this issue provided some evidence that deletions at chromosome 4q in a subset of CRCs may explain the loss of *UNC5C* expression [27]. In this report, based upon limited *in-vitro* data the authors suggested that epigenetic modifications of *UNC5C* may be responsible for downregulation of its expression, but these data precluded determination of whether such events occurred in a tumor-specific manner in CRC [27].

Given the apparent importance of the netrin pathway in the maintenance of gut epithelium, a better understanding of the molecular mechanisms that mediate loss of expression of these genes, and the consequence of combined losses of these receptors in a series of premalignant and malignant colonic tissues, is warranted. This thesis investigates these issues by analyzing a panel of CRC cell lines, adenomatous polyps (APs), and CRCs. Herein I report that the majority of CRCs demonstrate the loss of both NTN1 receptors. I also provide data suggesting that the inactivation of these receptors is mediated through both genetic and epigenetic mechanisms, and that these processes are orchestrated in a sequential manner during the evolution of multi-step colorectal carcinogenesis.

II. MATERIALS AND METHODS

1. Cell lines and primary CRC and AP tissues

Eight human colon cancer cell lines representing all the major pathways of genomic instability in CRC included Caco2, HT29, SW837,SW480, HCT116, LoVo, RKO and SW48 and CCD18Co (a colon fibroblast cell line), and were purchased from American Type Culture Collections (Manassas, VA). All cell lines were cultured in appropriate 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.

Tissue specimens of primary CRCs and their matched corresponding normal mucosa (CNM) from 147 patients who had undergone surgery were prospectively collected at the Okayama Saiseikai Hospital and Okayama University Hospital in Okayama, Japan. All CNM tissues were obtained from sites adjacent to, but at least 10 cm away from the original tumor. In addition, I obtained 52 colorectal adenomatous polyps (APs) from a different cohort at Chikuba Hospital in Okayama, Japan. All patients provided written informed consent and the study was approved by Institutional Review Boards of all institutions involved.

2. 5-Aza-2'-Deoxycytidine *(5azaD)* treatment

SW480 and HCT116 cells were plated at $2X10^5$ cells/100 mm culture dish. After 24 h of exponential growth, SW480 cells were treated with 5 μM *5azaD* (Sigma-Aldrich Inc., St. Louis, MO) and HCT116 cells were treated with 0.1 μM *5azaD* for 72 h respectively. Control cell lines were treated with equal volumes of PBS added to the culture medium. The culture medium containing *5azaD* was changed every 24 h. At the end of treatment periods, DNA and RNA were extracted 24 h post-treatment, and processed for analyses.

3. DNA and RNA extraction

Genomic DNA from the cell lines was extracted using QIAamp DNA mini kits (Qiagen, Valencia, CA). All CRCs, CNM, and six of the 52 adenomatous polyps were fresh-frozen tissue specimens, from which DNA was extracted using standard procedures that included proteinase-K digestion and phenol-chloroform extraction. DNA from the remaining 46 polyps, which were formalin-fixed, paraffin-embedded specimens, was extracted using Takara DEXPAT kits (Takara Bio Inc., Japan). Total RNA from cell lines was obtained using the TRIzol reagent (Life Technologies Inc. Carlsbad, CA).

4. Reverse transcription-polymerase chain reaction (RT-PCR)

The first strand cDNA synthesis was performed using Moloney murine leukemia virus reverse transcriptase (Invitrogen Life Technologies Inc., Carlsbad, CA) with a total of 1 μg RNA. RT-PCR was performed using *UNC5C*-specific primer pairs which have been previously described [28]. *β-actin* amplification was used as an internal control (Table 1). The PCR products were electrophoresed on a 2% agarose gel (ISC Bio Express, Kaysville, UT) and were imaged using a Gel Logic 200 Kodak Molecular Imaging System (Kodak, New Haven, CT).

Table 1. Primer Sequences for RT PCR, COBRA and bisulfite sequencing analysis

Assay	Primer name	Primer sequence	Product size (bp)
RT-PCR	RT-*UNC5C*-F	5'-TGCAAATGCTCGTGCTACCT-3'	258
	RT-*UNC5C*-R	5'-TGTGGTCCTTCTGATGAACCC-3'	
	RT-β actin-F	5'-TCACCATGGATGATGATATCGCC-3'	282
	RT-β actin-R	5'-CCACACGCAGCTCATTGTAGAAGG-3'	
COBRA	COBRA-F	5'-GTATTGGGAAAGAGGGATTTTTAAA-3'	157
	COBRA-R	5'-AACCCCACTAAACAAAAACTAAATC-3	
Bisulfite sequence	Bis-F	5'-GGTGTTTGTGTGTGTTTTTTATAGG-3'	336
	COBRA-R	5'-AACCCCACTAAACAAAAACTAAATC-3	

RT-PCR: reverse transcription PCR, COBRA: combined bisulfite restriction assay

5. Bisulfite modification and combined bisulfite restriction assay (COBRA)

Bisulfite modification of genomic DNA from CRC cell lines and clinical specimens was performed as described by Herman et al. [29]. I first identified tentative CpG islands in the promoter region of the *UNC5C* gene by mapping analysis using a CpG island search program (http://www.uscnorris.com/cpgislands/cpg.cgi). There were 42 CpG sites within the *UNC5C* promoter region investigated in this study (Figure 1). I subsequently analyzed the *UNC5C* methylation status using COBRA [30].

PCR amplification was performed using the primer sets COBRA-F and COBRA-R (Table 1 and Figure 1). Before initiating restriction endonuclease digestion, a small volume of PCR product was electrophoresed on 2% agarose gel to validate the specificity and efficiency of PCR. Thereafter, restriction enzyme digestion was carried out using BstUI (New England Bio Labs, Ipswich, MA). The percentages of fully methylated BstUI sites were calculated by determining the ratios between the BstUI-cleaved PCR product and the total amount of PCR product loaded.

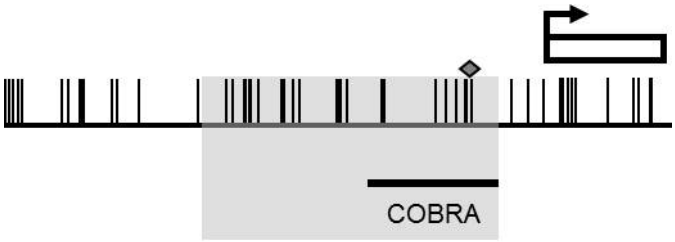


Figure 1. A schematic of the 5'-promoter region of the *UNC5C* gene: All CpG dinucleotide sequences are represented by vertical bars across the horizontal line depicting the promoter sequence. The solid line at the bottom right represents the specific region assayed by the combined bisulfite restriction assay (COBRA) to amplify nucleotides -216 to -60 of the promoter region (in which the start codon of *UNC5C* is defined as +1). The diamond on one of the vertical bars indicates the BstUI CpG restriction site. The gray rectangular square designates the region by bisulfite sequencing across nucleotide positions -395 to -60

6. Cloning and sodium bisulfite sequencing

Bisulfite-modified DNA from cell lines and clinical specimens was amplified using primer sets Bis-F and COBRA-R (Table 1). Each of the PCR products was subsequently cloned using the TOPO-TA cloning system (Invitrogen Life Technologies Inc., Carlsbad, CA). Nine to eleven white colonies indicating positive clones with PCR products were sequenced using ABI PRISM Big Dye Terminator v1.1 Cycle Sequencing Kits on an ABI PRISM 3100 Avant Genetic analyzer (Applied Biosystems, Foster city, CA)

7. Loss of heterozygosity (LOH) analysis

A set of three polymorphic microsatellite markers (D18S58, D18S64 and D18S69) was used to determine LOH at chromosome 18q. PCR amplifications were performed on genomic DNA templates from both tumor and CNM tissues using

fluorescently labeled primers. The amplified PCR products were electrophoresed on an ABI PRISM 3100 Avant Genetic analyzer and analyzed by GeneMapper fragment analysis software (Applied Biosystems, Foster City, CA). When comparing the signal intensities of individual markers in tumor DNA with that of the corresponding normal DNA, a reduction of at least 40% in the signal intensity was considered indicative of LOH.

8. Microsatellite instability (MSI), *KRAS* and V600E *BRAF* mutation analyses

MSI, *KRAS* and V600E *BRAF* mutation analyses data were available for all 147 patients from previous study [31].

9. Statistical analysis

Statistical analyses were done using JMP software (version 4.05J; SAS Institute, Inc., Cary, NC). First, *UNC5C* methylation levels were analyzed as a continuous variable, as were computed means, standard error of the means (S.E), and standard deviations (S.D). Next, the methylation status of the *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's exact test, χ^2-test or a Wilcoxon/Kruskal-Wallis's test. The associations between *UNC5C* promoter methylation and clinico-pathological variables were analyzed using χ^2-tests. All reported *P* values were 2-sided and a *P* value <0.05 was considered statistically significant.

III. RESULT

1. Hypermethylation of *UNC5C* induces transcriptional silencing in CRC cell lines

Using RT-PCR, I examined *UNC5C* gene expression in the panel of eight CRC cell lines and CCD18Co cells. All CRC cell lines (but not CCD18Co) lacked expression of *UNC5C* gene transcripts (Figure 2). To determine the mechanism for loss of UNC5C expression, I measured the methylation status of the *UNC5C* promoter by COBRA, and observed that all CRC cell lines had hypermethylation of the *UNC5C* promoter. Contrariwise, CCD18Co cells, which expressed *UNC5C*, did not show *UNC5C* promoter methylation (Figure 3A). To confirm COBRA results, I performed bisulfite sequencing in all cell lines. Most cancer cell lines were heavily methylated at individual CpG dinucleotides, while CCD18Co was unmethylated in each sequenced clone (Figure 3B).

I tested the hypothesis that epigenetic silencing caused the loss of *UNC5C* expression in cultured CRC cells by treating SW480 and HCT116 cells with the demethylating agent, *5azaD*. As anticipated, *5azaD* treatment restored *UNC5C* mRNA expression, and this was associated with significant demethylation of the promoter region of *UNC5C* by COBRA (Figure 4).

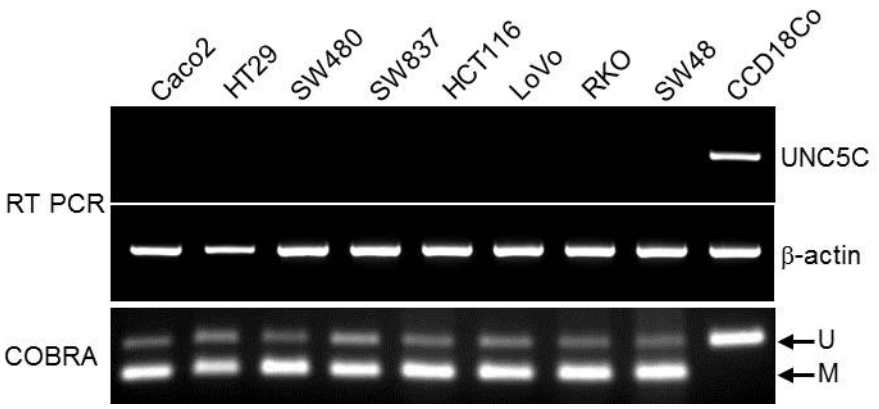
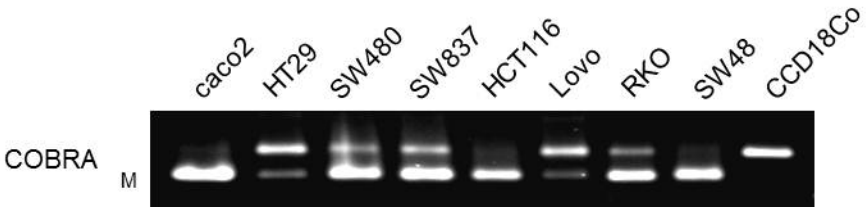


Figure 2. Correlation of *UNC5C* hypermethylation and loss of *UNC5C* mRNA expression in CRC cell lines: Eight CRC cell lines and CCD18Co cells were analyzed for mRNA expression by RT-PCR of the *UNC5C* and *β-actin* genes. The lowest panel illustrates the methylation profile obtained from COBRA. An arrow with 'M' indicates the methylated DNA product resulting from BstUI-digestion, while 'U' corresponds to the unmethylated band.

A.



B.

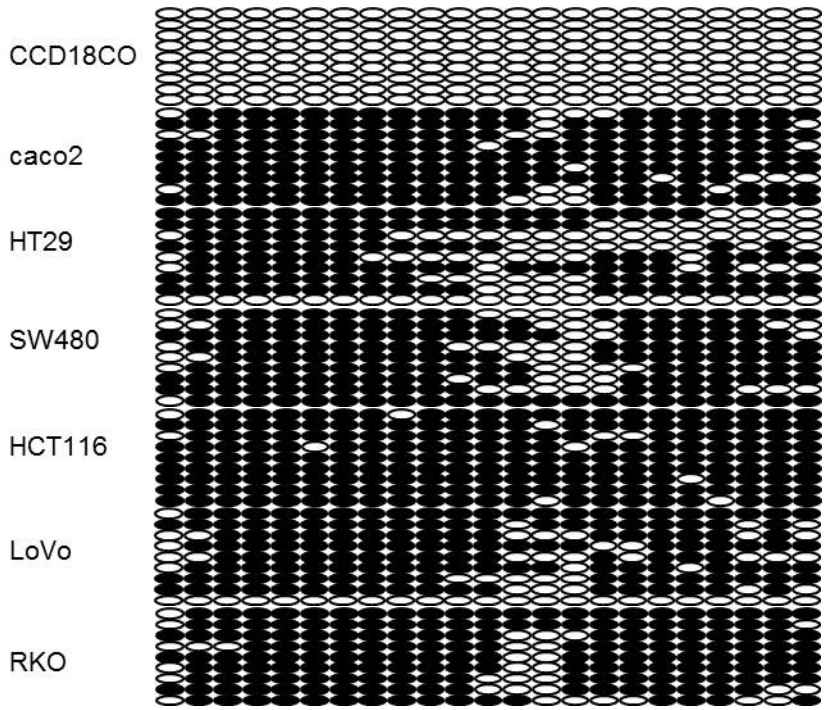


Figure 3. COBRA and Bisulfite sequencing of CRC cell lines: The results using 8 cell lines for COBRA and bisulfite sequencing analysis are shown in Figure A and B, respectively. 'M' in Figure 3A indicates methylated DNA products following BstUI digestion. For bisulfite sequencing analysis, PCR products were cloned into a TOPO cloning vector and sequenced. For each cell line, at least 9 clones were sequenced. Empty circles indicate unmethylated CpG sites, while filled circles represent methylated CpG sites.

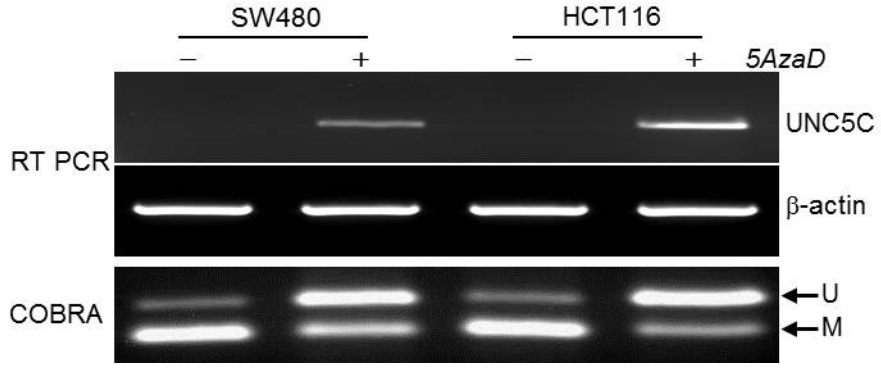


Figure 4. 5-Aza-2'-Deoxycytidine *(5AzaD)* treatment restores *UNC5C* expression in CRC cell lines: SW480 and HCT116 were treated with *5AzaD* at 5 μM and 0.1 μM concentrations respectively, for 72 h. (-) mock-untreated; (+) *5AzaD* treated

2. *UNC5C* is frequently hypermethylated in colorectal tissues

I tested the hypothesis that *UNC5C* methylation is a common event in CRC by investigating its methylation status in a cohort of 147 CRCs and CNM tissue specimens, and a group of 52 APs. A panel of representative COBRA results is depicted in Figure 5 and Figure 6. I analyzed these results using mean *UNC5C* methylation levels as continuous variables. Using a cut-off value of >1% methylation as a criterion for positive methylation, I observed that the *UNC5C* promoter was hypermethylated in 77.6% (114/147) of CRCs, 63.5% of (33/52) APs, and 14.3% (21/147) of CNMs. The overall degree of *UNC5C* methylation was significantly higher in CRCs and APs compared to CNMs (34%$\pm$22% [SD] in CRCs, 56%$\pm$33% in APs, and 4.7%$\pm$2.1% in CNMs $P<.0001$, Wilcoxon/Kruskal-Wallis test). Using these results, I optimized the cut-off value for *UNC5C* methylation at 5% (i.e., $\geq$5% methylation = positive and <5% methylation = negative). Employing this criterion, I observed significant aberrant hypermethylation of *UNC5C* in 76.2% of CRCs (112/147) and 63.5% of APs (33/52) compared to 6.1% of CNMs (9/147) ($P <.0001$, χ^2-test). Of note, even among APs, *UNC5C* methylation was limited to the advanced adenomas (33/46), and was not present in any of the smaller, non-advanced polyps (0/6).

Methylation data obtained by COBRA were confirmed with bisulfite sequencing analysis. Sequencing data revealed dense methylation of CpG islands in CRCs, but showed no evidence for this epigenetic change in the CNM (Figure 5). Data from APs were similarly confirmed (Figure 6). Collectively, these data indicate that *UNC5C* expression is frequently lost or down-regulated in colorectal neoplasms, and that this event is associated with the aberrant methylation of the promoter region of *UNC5C* gene.

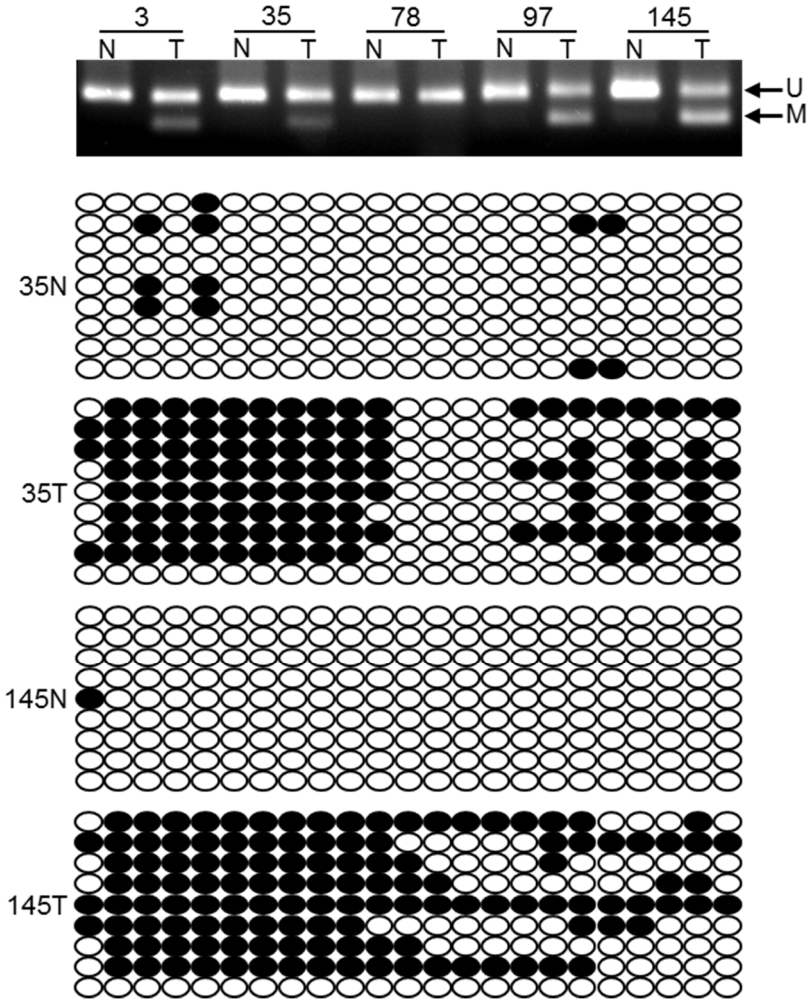


Figure 5. COBRA and bisulfite sequencing analysis of *UNC5C* in CRCs: Representative results for COBRA and bisulfite sequencing analysis are shown in the upper and lower panels respectively. The arrow marked 'M' in the top panel indicates methylated DNA products following BstUI digestion, while 'U' represents unmethylated alleles.

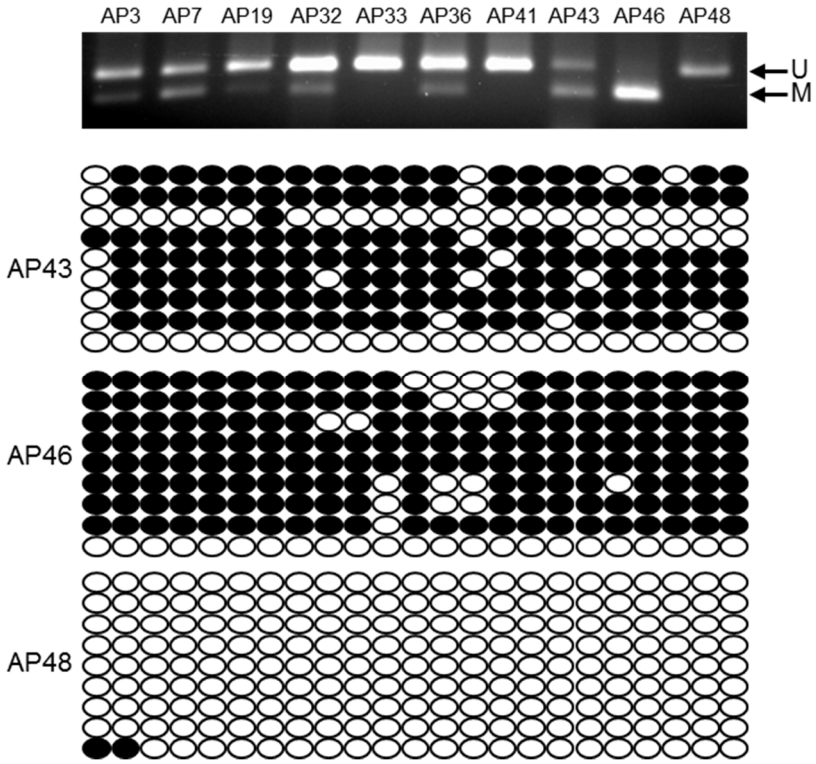


Figure 6. COBRA and bisulfite sequencing analysis for *UNC5C* in APs: This figure illustrates representative examples of data obtained from COBRA and bisulfite sequencing in polyps.

3. *UNC5C* methylation is associated with larger polyp size, older age, tumor grade and histology, as well as mutant *BRAF*

I next examined associations between *UNC5C* promoter methylation status as a categorical variable and the clinicopathologic and genetic features of patients with APs and CRCs (Table 2 and Table 3). I found significantly more *UNC5C* methylation in larger versus smaller polyps (13.7 mm versus 11.4 mm, *P =.02*, *Wilcoxon/ Kruskal-Wallis* test, Table 2), whereas there were no significant associations among any of the other variables.

While performing similar analyses in CRCs (Table 3), I found that *UNC5C* methylation was significantly more frequent in elderly (≥ 65 years) compared to younger (<65 years) patients (P= 0.008 χ^2-test). However, no significant differences were found when patient gender, tumor location or tumor stage were compared in methylated versus unmethylated CRCs. Additionally, I found that *UNC5C* methylation was significantly greater in well differentiated, or poorly differentiated mucinous CRCs compared to moderately differentiated CRCs (P=0.002 χ^2-test). *UNC5C* methylation also correlated with MSI, although these results were not statistically significant due to the smaller number of MSI-H CRCs investigated in this study. V600E *BRAF* mutation was associated with significantly greater *UNC5C* methylation compared to the subset of CRCs that were wild-type (P=0.006 χ^2-test; Table 3). Methylation data obtained by COBRA were confirmed with bisulfite sequencing analysis. Sequencing data revealed dense methylation of CpG islands in CRCs, but showed no evidence for this epigenetic change in the CNM (Figure 5). Data from APs were similarly confirmed (Figure 6). Collectively, these data indicate that *UNC5C* expression is frequently lost or down-regulated in colorectal neoplasms, and that this event is associated with the aberrant methylation of the promoter region of *UNC5C* gene.

Table 2. Relationships between *UNC5C* methylation and clinicopathologic features in patients with adenomatous polyps

		UNC5C methylation Status No. (%)		
		Methylated*	Unmethylated	*P-value*
Age	≥ 65 (n=19)	13 (68)	6 (32)	0.57 [a]
	< 65 (n=33)	20 (61)	13 (68)	
Gender	Female (n=10)	7 (70)	3 (30)	0.63 [a]
	Male (n=42)	26 (62)	16 (38)	
Location	Proximal (n=16)	10 (63)	6 (38)	0.92 [a]
	Distal (n=36)	23 (64)	13 (36)	
Histology	TA (n=40)	24 (60)	16 (40)	0.34 [a]
	TA with villous (n=12)	9 (75)	3 (25)	
Size	Mean diameter (95%CI)	13.7 mm (12.0 - 15.2)	11.4 mm (9.2 -13.5)	.02[b]

*Methylated cases were categorized as those with ≥5% promoter methylation.

[a]*P-values* were calculated using the χ^2-test.

[b]*P-value* was calculated using the *Wilcoxon/ Kruskal-Wallis* test.

TA: tubular adenoma; TA with villous: TA with villous features

Table 3. Relationships among *UNC5C* methylation and clinicopathologic characteristics in patients with colorectal cancer

		UNC5C methylation Status No. (%)		
		Methylated*	Unmethylated	*P-value*
Age	≥ 65 (n=79)	67 (85)	12 (15)	0.008
	< 65 (n=68)	45 (66)	23 (34)	
Gender	Female (n=50)	38 (76)	12 (24)	0.97
	Male (n=97)	74 (76)	23 (24)	
Location	Proximal (n=41)	30 (73)	11 (27)	0.59
	Distal (n=106)	82 (77)	24 (23)	
Tumor stage (Dukes')	A (n=26)	23 (81)	5 (19)	0.65
	B (n=42)	34 (81)	8 (19)	
	C (n=49)	36 (73)	13 (27)	
	D (n=30)	21 (70)	9 (30)	
Histology	well (n=26)	25 (96)	1 (4)	0.002
	Mucinous/poor (n=11)	10 (91)	1 (9)	
	moderate (n=110)	77 (70)	33 (30)	
MSI status	High (n=4)	4 (100)	0 (0)	0.08
	Low (n=43)	36 (84)	7 (16)	
	Stable (n=100)	72 (72)	28 (28)	
Ras pathway	*BRAF* mut (n=4)	4 (100)	0 (0)	0.006
	K-RAS mut (n=45)	40 (89)	5 (11)	
	Wild type (n=98)	68 (69)	30 (31)	

*Methylated cases were categorized as those with ≥5% promoter methylation. MSI: microsatellite instability; Mut: mutation

P-values were calculated by χ^2-test.

4. Deletions at the 18q/DCC locus are frequent in CRCs

Since the primary mechanism that accounts for loss of *DCC* in CRC is deletions in chromosome 18q [11, 32, 33], I determined the frequency of allelic losses in a panel of LOH markers that mapped closely to *DCC*. Of the 147 specimens investigated, 70.1% (103/147) of cases yielded informative analyses at these three loci. Among the informative cases, 18q losses were frequent, and 61.2% (63/103) of the CRCs showed allelic imbalance in at least one of the markers (Figure 7).

I tested the hypothesis that *DCC* losses and *UNC5C* methylation occur concurrently, versus independently of one another. I discovered that 48.5% (50/103) of CRC cases had undergone simultaneous methylation of *UNC5C* and LOH of *DCC* (Figure 7). Independent *UNC5C* promoter methylation or 18q loss alone was also present in 28.2% (29/103) and 12.6% (13/103) of CRCs respectively. Considered together, alterations in *UNC5C* and *DCC* individually or concurrently were present in 89.3% (92/103) of CRCs, and only 10.7% (11/103) of tumors lacked evidence for either of these defects.

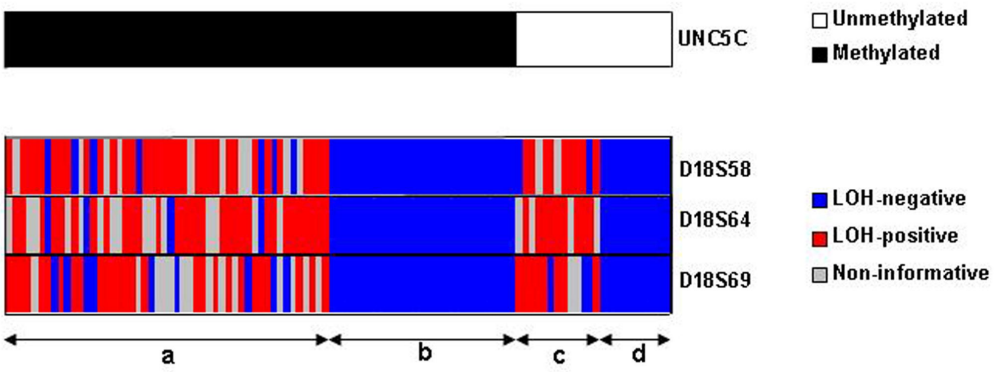


Figure 7. Methylation profile of *UNC5C* and LOH of 18q loci: Among the 147 CRC analyzed for LOH, 103 cases were informative. The upper horizontal bar depicts the methylation status of *UNC5C* in 103 cancers; the filled (black) portion on the left indicates methylated cases, and the unfilled (white) portion on the right indicates unmethylated CRCs. The three lower bars represent LOH results. Red indicates CRCs with LOH at that marker; blue indicates no LOH, and gray represent non-informative cases. Below the bars, "a" indicates CRCs that had simultaneous *UNC5C* methylation and *DCC* deletion (50/103, 48.5%); "b" represents CRCs that showed only *UNC5C* methylation (29/103, 28.2%); "c" denotes cases with exclusive 18q LOH (13/103, 12.6%); and "d" represents the cancers that had neither alteration (11/103, 10.7%).

5. *UNC5C* methylation occurs early, while *DCC* loss occurs in advanced stage CRCs

Since *UNC5C* and *DCC* both serve as dependence receptors for NTN1, I asked whether defects in these receptors accumulate in a systematic manner, or stochastically, during the development of progressive colorectal neoplasia. Therefore, I looked for associations between *UNC5C* and/or *DCC* defects and Dukes' tumor stage in the 103 CRCs which were informative for both *UNC5C* methylation and *DCC* LOH. As shown in Figure 8**,** concurrent alterations in the *UNC5C* and *DCC* genes were more commonly observed in advanced stage CRCs (60%, 48% and 50% for stages B, C and D respectively) than in earlier stage cancers (25% for stage A), although this association did not achieve statistical significance, perhaps due to the small sample size of each stage.

Segregating tumors based upon individual defects in either *UNC5C* or *DCC* and their relationship with tumor stage, I found that methylation-induced silencing of *UNC5C* by itself was significantly more frequent in Dukes' stage A neoplasms, and became progressively less in later stage CRCs (Stage A: 56%, B: 21%, C: 27% and D: 18%; $P = 0.046$, χ^2-test). Supporting the finding that *UNC5C* methylation may be an earlier event in the development of colorectal neoplasia, I observed a similar trend for a higher frequency of *UNC5C* methylation in APs (63.5%). Contrariwise, the frequency of *DCC* loss was inversely correlated with early tumor stage. I observed that allelic imbalances at 18q loci demonstrated a gradual increase with advancing Dukes' stage, although these data did not reach significance (stages A: 6.2%, B: 7.1%, C: 13.5%, and D: 22.7%; $P = 0.39$, Fisher's exact test).

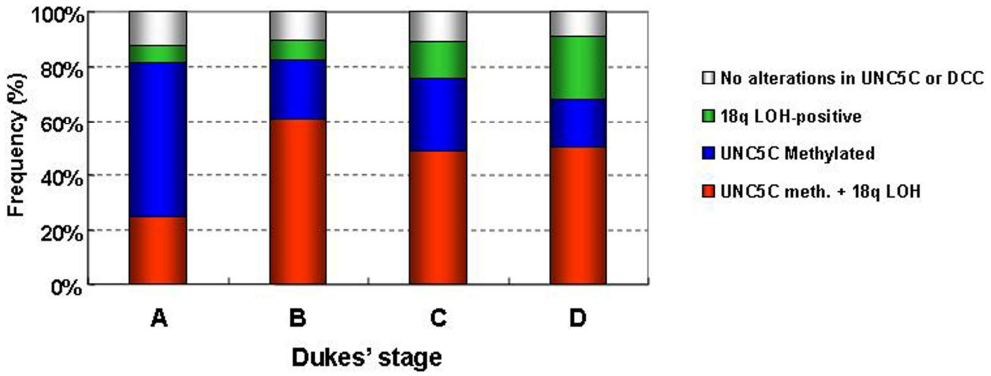


Figure 8. Correlation of *UNC5C* methylation, *DCC* deletions and their relationship with tumor stage: The Y-axis represents the prevalence of *UNC5C* methylation and/or 18q LOH, and the X-axis groups tumors based upon Dukes' stage.

IV. DISCUSSION

This study investigated the molecular events responsible for abrogation of the netrin pathway in CRC, and the role played by the two dependence receptors, *UNC5C* and *DCC*. I analyzed a large series of colorectal tissues that included 52 APs, 147 CRCs with adjacent normal mucosa, and a panel of eight CRC cell lines. I found that both *UNC5C* and *DCC* are inactivated in nearly 90% of CRCs, and that these alterations occur through genetic and epigenetic processes. *UNC5C* inactivation in CRC is primarily a consequence of hypermethylation of its promoter, while the loss of *DCC* is principally due to deletions at chromosome 18q. I also provide evidence that the timing of molecular alterations in *UNC5C* and *DCC* is not random, as *UNC5C* inactivation occurs in earlier neoplastic lesions, while LOH of *DCC* loci accrue progressively through multistep colorectal carcinogenesis.

The recent discovery of a 'dependence receptor' concept suggests that receptors can be functionally important even when not bound by their ligands, which has helped to clarify some of the contentious issues in colorectal tumorigenesis [18]. *DCC* and *UNC5C* both serve as dependence receptors for NTN1; these receptors transmit cell death signals in the absence of ligand [27, 34, 35], and the binding of NTN1 to these two receptors inhibits apoptosis [15, 18]. Multiple studies have highlighted the functional role for UNC5H members in human carcinogenesis [36-38], and a recent study elegantly demonstrated a tumor suppressor role for UNC5C in the colon, wherein ectopic expression of UNC5C in cell lines lacking endogenous UNC5C resulted in the suppression of anchorage-independent growth and an inhibition of Ras-dependent cell invasion [27]. The mechanistic details underlying the loss of dependence receptors in human cancers are still unclear, but when these receptors are down-regulated or deleted, this impairs adequate levels of programmed cell death, and provides the malignant cells with selective growth advantage. I sought a better understanding of the molecular events that lead to the inactivation of these dependence receptors.

A prior study reported that 77% of CRCs had experienced loss of *UNC5C* expression [27]. It was suggested that loss of *UNC5C* was due to allelic losses of chromosome 4q, and mutations were rarely observed [27]. Allelic losses at 4q have been previously reported in several human cancers, but the frequencies have ranged from 23-39% [27, 39, 40]. Even if it were assumed that every 4q loss caused loss of *UNC5C* expression in CRC, this mechanism alone would not account for the *UNC5C* under-expression in CRC. I performed LOH analyses using the markers suggested in the previous study, but could not confirm those findings, in part due to non-informativity of the markers (data not shown).

I then hypothesized that loss of *UNC5C* expression in CRC may be mediated through aberrant methylation of its promoter. I first identified the critical CpG island in the *UNC5C* promoter that associates with loss of gene expression in CRC cell lines. I found that most colon cancer cell lines lacked *UNC5C* expression, and were methylated in this region. To further confirm that methylation was the cause of *UNC5C* silencing, I demonstrated restoration of *UNC5C* expression in the cell lines using the demethylating agent, *5azaD*.

I then determined that epigenetic silencing of *UNC5C* is commonly present in CRC and AP tissues. I found evidence for frequent *de novo* methylation of *UNC5C* in both advanced APs and CRCs, which was rarely observed in normal colonic epithelium. The presence of *UNC5C* methylation in advanced APs is relevant, since the risk for advanced adenomas to progress to cancer is much higher than for smaller, early polyps [41, 42]. I could not perform UNC5C immunochemistry on the clinical materials due to the unavailability of an adequate antibody. However, I believe this experiment may not be critical, since methylation of the promoter region investigated demonstrated a perfect association with loss of expression in the cancer cell lines. Since *UNC5C* methylation was very frequent in CRC, and associated with MSI as well as mutant BRAF, these data suggest that *UNC5C* methylation may be a CIMP-sensitive and tumor-specific marker, but may not be CIMP-specific since it would far exceed the reported CIMP frequencies in these

cancers [2, 43]. Although future studies will be required to interrogate this issue in more detail, our data clearly demonstrate that *UNC5C* methylation occurs frequently, and occurs in a tumor-specific manner.

The second netrin receptor, *DCC*, was originally discovered as a putative tumor suppressor gene in CRC [11]. *DCC* resides on chromosome 18q, which is the most commonly deleted chromosomal region in colorectal neoplasia [10, 11, 32, 33]. Methylation induced silencing of *DCC* has been suggested, but the evidence had been inconclusive [44, 45]. A tumor suppressor role for *DCC* has been questioned in studies which failed to demonstrate a clear malignant phenotype in *DCC* knockout mice models [13]. However, more recent studies have challenged this and favored a role for *DCC* in suppressing tumor growth and metastasis [15-17]. The recent proposition that *DCC* serves as a dependence receptor for *NTN1* has rejuvenated the notion that *DCC* functions as proapoptotic growth suppressor when not bound by its ligand [23, 34, 35]. In the gastrointestinal tract, *NTN1* performs an important role in the maintenance and renewal of intestinal epithelium by regulating cell survival or cell death through its interaction with *UNC5C* and *DCC* [15, 23, 24]. Any abrogation of the netrin pathway, including dysregulation in *NTN1* or its two receptors, may inhibit apoptosis, which would be permissive of neoplasia.

Since both *DCC* and *UNC5C* share the same netrin ligand and are co-localized in the gut [15, 19, 25, 27], I reasoned that the solitary inactivation of either *DCC* or *UNC5C* alone might not be sufficient to promote tumor development in the colon. In this study, I found that a large majority of CRCs demonstrated simultaneous alterations in both *DCC* and *UNC5C*; supporting our contention that inactivation of both receptors may be required in the evolution of CRC. Although a lack of sufficient material prevented us from performing LOH analyses for *DCC* (18q) in the APs, previous studies have indicated that *DCC* losses probably are quite uncommon in early premalignant lesions, and accumulate only at the later stages of CRC development [32, 46, 47].

My finding that dysregulation of *UNC5C* predominantly occurs in the early phase of colorectal neoplasia while *DCC* loss occurs later suggests that inactivation of these receptors is not a random process, but occurs in a statistically predictable, sequential manner. These data are consistent with the observation that epigenetic alterations are frequently present in adenoma progression [48], perhaps because methylation-induced silencing results in gene silencing, which has a more predictable outcome than point mutations or chromosomal rearrangements.

IV. CONCLUSION

In conclusion, I provide the evidence that most CRCs have alterations in both *UNC5C* and *DCC* netrin receptors. While *UNC5C* expression is primarily regulated via epigenetic regulation, *DCC* defects are mediated through allelic deletions. Additionally, the timing of these molecular alterations in the dysregulation of the NTN1 pathway is not random, with *UNC5C* inactivation occurring earlier, and *DCC* losses followed in the later stages of colorectal carcinogenesis. I propose that the netrin-1 pathway plays an important role in adenoma-to-carcinoma sequence in the colon.

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ABSTRACT (IN KOREAN)

대장암에서의 Netrin-1 수용체들의 변이에 관한 고찰분석

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신 성 관

대장-직장암은 상피 세포의 성장과 분화 등을 조절하는 여러 유전자의 유전적 그리고 epigenetics 적 변화가 축적되어 발생한다. 현재까지 3 가지의 기전이 대장-직장암의 형성에 기여한다고 알려져 있는데 이는 현미부수체 불안정성 (microsatellite instability, 이하 MSI), 염색체 불안정성 (chromosomal instability, 이하 CIN) 그리고 CpG Island Methylator Phenotype (이하 CIMP)이다. 대장-직장암에서 CIN 의 공통적 특징은 *APC* 유전자와 연관된 염색체 5q, *p53* 가 위치하는 17p, 그리고 *DCC/SMAD* 유전자가 존재하는 18q 에서 이형 접합자 소실 (loss of heterozygosity, 이하 LOH)이 흔히 관찰되는 것이다. 특히 대장-직장암에서 18q 가 흔히 소실됨이 최초로 밝혀진 후 이 염색체에 존재하는 *DCC* 유전자는 종양 억제 유전자로서 조명을 받게 된다. 또한 근래 *DCC* 와 *UNC5H* 가 *Netrin-1* (NTN1)에 대한 '의존적 수용체' (dependence receptors)로서 작용한다는 가설은 *DCC* 의 종양 억제 유전자로서의 논란에 새로운 불씨를 지폈다. 리간드인 NTN1 은 뇌를 포함한 여러 조직에 존재하는 laminin-related 단백질이며, *DCC* 와

UNC5H 에 결합하여 작용을 나타낸다. 전통적 수용체-리간드의 개념과는 달리 '의존적 수용체'는 리간드가 결합하지 않은 경우에는 세포 자연사를 유도하는 역할을 하지만, 리간드가 결합하는 경우에는 세포 증식, 분화, 이동 등의 신호를 매개하여, 리간드의 존재여부에 의존적으로 상반된 역할을 수행할 수 있다는 의미로 상기와 같은 명칭을 얻게 되었다. 즉 NTN1 이 고농도로 분포할 때는 장 점막의 유지, 장 상피 세포의 이동, 조직 재생 등에 관여하지만, NTN1 의 농도가 낮은 villus tip 에서는 *DCC* 와 *UNC5C* 두 수용체가 세포 자연사를 촉진하여 장 점막 세포의 탈락을 유발하여 장 점막의 항상성 유지에 기여는 것이다.

전술한 바와 같이 NTN1 은 *UNC5H* 수용체 군의 리간드이기도 하다. 이 군에 속하는 수용체들로는 *UNC5A*, *UNC5B*, *UNC5C* 그리고 *UNC5D* 가 있으며 흥미로운 사실은 이 수용체 군이 여러 암 종에서 그 표현이 저하 또는 소실되어 있다는 점이다. 특히 수용체 중의 하나인 *UNC5C* 의 발현이 대장-직장암에서 현저히 소실되어 있다. 일부 밝혀진 UNC5C 수용체의 소실 기전은 대장-직장암의 일부에서 이 유전자가 존재하는 염색체 4q 의 LOH 기는 하지만 아직 이 유전자의 소실의 기전은 잘 알려져 있지 않다.

NTN1 경로가 장 점막의 항상성 유지에 중요한 역할을 수행한다는 여러 연구 결과들을 종합해 볼 때 이와 관련된 *DCC* 와 *UNC5C* 소실을 매개하는 분자 생물학적 기전을 규명하고 이 두 유전자의 소실이 전암 병변에서 암종으로의 전환으로 대표되는 대장-직장암의 발암 기전에서 어떤 역할을 할 것인가에 대한 연구가 필요함은 자명하다고 할 수 있다. 대장.직장암 세포주, 선종성 용종 그리고 대장.직장암 조직의 분자 생물학적 분석을 통해 이러한 명제들에 대한 의문을 풀기 위해 본 연구를 수행하였다.

8 개의 대장-직장암 세포주 (Caco2, HT29, SW837,SW480, HCT116, LoVo, RKO, SW48)를 이용하여 *UNC5C* 유전자의 발현 유무와 promoter 과메틸화의 관계를 알아보고, 이러한 상관 관계가 147 명의 산발성 대장-직장암 환자로부터 채취된 암 조직과 정상 점막 조직, 그리고 52 개의 선종성 용종의 임상 조직에도 적용될 수 있는 지 분석하였다. 대장-직장암 조직에서 염색체 18q 의 LOH 를 분석하여 *DCC* 유전자의 소실을 측정하였으며 이상의 실험을 통해 얻어진 결과를 분자 생물학적, 임상 병리적 특징과 함께 분석하였다.

연구된 모든 대장-직장암 세포주에서 *UNC5C* promoter 의 과메틸화가 관찰되었고 이는 *UNC5C* 의 소실과 연관이 있었으며, *5-Aza-2'-deoxycytidine* 의 처리를 통해 유전자의 재발현이 관찰되었다. 임상조직의 분석에 있어서는 대장-직장암의 76.2%, 선종성 용종의 63.5%에서 과메틸화가 관찰되었지만 정상 조직에서는 6%만이 과메틸화를 보여 통계적으로 유의한 차이를 보여주었으며 ($p < 0.0001$) 이 결과를 통해 대장-직장암에서 *UNC5C* 소실의 주 기전은 promoter 과 메틸화에 의함을 알 수 있었고 이는 종양 특이적 현상임을 밝혀 내었다. 대장-직장암 조직의 61%에서 염색체 18q 의 소실을 관찰할 수 있어서, 이 결과를 *UNC5C* 의 epigenetics 적 자료와 같이 분석 할 때, 약 90%의 대장-직장암에서 의존적 수용체의 소실이 있음을 알 수 있었다. 흥미롭게도 *UNC5C* 의 과메틸화는 조기 병변 (용종, 조기 대장-직장암)에서 두드러지게 관찰된 반면, *DCC* 의 소실은 진행성 암종에서 흔하게 나타났다.

대부분의 대장-직장암에서 NTN1 수용체의 결손이 관찰된 것은 이 성장 조절 경로가 발암 기전에서 매우 중요한 역할을 함을 시사한다고 할 수 있다. 그리고 *DCC* 와 *UNC5C* 두 유전자의 소실이 유전학적 그리고 epigenetics 적 변이에 의함을 밝혀내었고, 더욱이 대장-직장암의 다

단계적 발생에 있어서, 두 유전자의 변이가 무작위적으로 발생하는 것이 아니라 순차적으로 조율됨을 처음으로 증명하였다.

핵심되는 말: UNC5C, DCC, 대장-직장암, promoter 과메틸화, 현미부수체 불안정성,의존적 수용체