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Aberrant epigenetic modifications of LPHN2 function as a potential cisplatin-specific biomarker for human gastrointestinal cancer

위장관암에서 Cisplatin에 특이적인
바이오마커로서 LPHN2의 비정상적인
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서울대학교 융합과학기술대학원
분자의학 및 바이오제약학과
전 미 성

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지도교수 김 태 유
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서울대학교 융합과학기술대학원
분자의학 및 바이오제약학과
전미성

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위 원 장 강경희 (인)
부 위 원 장 김 태 유 (인)
위 원 원 엄 석 아 (인)

Aberrant epigenetic modifications of LPHN2 function as a potential cisplatin-specific biomarker for human gastrointestinal cancer

by

Mi-Seong Jeon

(Directed by Tae You Kim, M.D., Ph.D.)

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Department of Molecular Medicine and Biopharmaceutical Sciences
World Class University Graduate School of
Convergence Science and Technology
Seoul National University

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Approved by thesis committee :

Professor Gyeong Hoon Kang Chairperson

Professor Tae you Kim Vice Chairperson

Professor Seok Ah Im

ABSTRACT

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Mi-Seong Jeon
Department of Molecular Medicine
and Biopharmaceutical Sciences
World Class University Graduate School
of Convergence Science and technology
Seoul National University

Epigenetic alterations, such as DNA methylation and histone modifications, are considered common mechanisms that contribute to aberrant gene expression during tumorigenesis. Recently, epigenetic alterations of specific genes have been identified as

diagnostic biomarkers for human cancers. However, there are currently no standardized epigenetic biomarkers for drug sensitivity in human gastrointestinal cancer. Here I report that in human gastric and colon cancers, Latrophilin2 (*LPHN2*) is silenced by epigenetic modifications, including CpG island methylation and aberrant histone modifications. Treatment with a DNA methyltransferase inhibitor induced demethylation of the methylated *LPHN2* CpG island and altered histone modifications around the *LPHN2* promoter, thereby restoring *LPHN2* expression. In addition, I confirmed that *LPHN2* was silenced by DNA hypermethylation in primary gastric and colon tumor tissues compared to their normal counterparts. Interestingly, I found that cancer cells with methylated *LPHN2* exhibited higher sensitivity to cisplatin. Also, 5-Aza-2′-deoxycytidine (5-aza-CdR) combined with cisplatin decreased the cytotoxicity of cisplatin in cancer cells with methylated *LPHN2*. Moreover, *LPHN2* knockdown in cancer cells with high *LPHN2* expression sensitized these cells to the anti-proliferative effects of cisplatin. Because the *LPHN2* methylation status was not responsible for differential sensitivity to other chemotherapeutic drugs, the methylation status

of *LPHN2* might confer sensitivity to cisplatin in human gastric and colon cancers. Taken together, my data suggest that epigenetic alterations induce transcriptional inactivation of *LPHN2,* which intensifies the response to cisplatin in human gastric and colon cancers. Thus, the methylation status of *LPHN2* is a potential novel epigenetic biomarker for cisplatin treatment in human gastric and colon cancers.

Keywords : Epigenetics, DNA methylation, histone modification, *LPHN2*, chemosensitivity

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INTRODUCTION

Aberrant gene function and altered patterns of gene expression are typical features among various cancers [1]. In particular, epigenetic modifications, which are defined as heritable changes in gene expression that are not caused by changes in the DNA sequence, are increasingly identified as hallmarks of human cancers [1,2,3,4]. DNA methyltransferases (DNMTs) hypermethylate CpG islands, resulting in transcriptional silencing of various genes [5,6]. DNMTs catalyze the transfer of a methyl group to the carbon 5 position of cytosine residues in CpG dinucleotides [7]. In addition to DNA methylation, histone modifications also help organize the nuclear architecture to dynamically regulate gene expression and gene activities [6,8]. Thus, DNA methylation cooperates with histone modifications to silence tumor suppressor genes during tumorigenesis [9].

The incidence and mortality of gastrointestinal cancers, including gastric and colon cancer, has increased globally [10,11,12]. Recent genome-wide epigenomic modification studies have reported various genes that are frequently inactivated by epigenetic modifications in gastric and colon cancer [13,14].

Interestingly, epigenetic modifications are being recognized as new therapeutic targets for the treatment and prognosis of gastrointestinal cancers [10,11,15,16]. However, there are no standardized epigenetic markers that can serve as novel indicators for the chemosensitiity of human gastrointestinal cancer [17,18,19].

In this study, I found *LPHN2*, a member of the latrophilin subfamily of G-protein coupled receptors [8,20,21], is transcriptionally inactivated by DNA methlyation and histone modifications in human gastric and colon cancer cells. Cancer cells with methylated *LPHN2* may serve as a novel epigenetic biomarker for cisplatin chemosensitivity. The *LPHN2* mehtylation status does not confer differential sensitivity to other chemotherapeutic agents; therfore, I propose that epigenetic modification of *LPHN2* is a promising cisplatin-specific biomarker.

MATERIALS AND METHODS

1. Cell culture and Primary human tissues

Eleven human gastric cancer cell lines and 15 human colon cancer cell lines were obtained from the Korea Cancer Cell Bank (Seoul, South Korea). Cells were cultured in RPMI 1640 media supplemented with 10% fetal bovine serum (FBS) and gentamicin (10 μg/mL)[22]. HCT116 and DKO cells, which were generous gifts from Dr. B. Vogelstein (Johns Hopkins University) [23], were cultured in McCoy's 5A media.

Twenty-four primary human gastric tumor tissues, ten primary human colorectal tumor tissues, and their matching normal tissues were obtained from Seoul National University Hospital. The study protocol was reviewed and approved by the Institutional Review Board of Seoul National University Hospital. After surgical removal, tissues were immediately frozen in liquid nitrogen and stored at −80℃ until use.

2. RT-PCR and quantitative real-time RT-PCR

Total RNA was prepared using the TRI Reagent (Molecular Research Center) in accordance with the manufacturer's instructions as previously described [24]. Briefly, cDNA was synthesized from 2 μg of total RNA using ImProm-II reverse transcriptase (Promega) and amplified by RT-PCR using HotStart Taq (Qiagen) and gene-specific primers. 18S ribosomal RNA was used as an internal RT-PCR control. For quantitative real-time RT-PCR (qRT-PCR), cDNAs were amplified using Premix Ex Taq (TaKaRa), SYBR Green I (Molecular Probes), and a Step One Plus system (Applied Biosystems).

3. Bisulfite genomic sequencing analyses and pyrosequencing

Genomic DNA (gDNA) was isolated using the QIAamp DNA mini kit (Qiagen), and 1 μg gDNA was modified using the EpiTect Bisulfite kit (Qiagen) according to the manufacturer's instructions [12]. Sodium bisulfite-modified DNA was amplified using specific primer sets. PCR products were gel-purified and cloned into TA cloning vectors (RBC Bioscience). Inserted PCR fragments of individual clones were then sequenced. For pyrosequencing analyses, bisulfite-modified gDNA was amplified using biotinylated primers specific for the *LPHN2* CpG island. Preparation of single-stranded DNA template, annealing to the pyrosequencing primer, and pyrosequencing were performed using PyroGold Q96 SQA reagents with a PyroMark ID pyrosequencer (Qiagen) according to the manufacturer's protocol. Pyrosequencing was performed using PyroGold Q96 SQA reagents with a PyroMark ID pyrosequencer (Qiagen) according to the manufacturer's protocol [12].

4. Chromatin immunoprecipitation (ChIP)

ChIP assays were performed as previously described [25]. Differences in DNA enrichment for ChIP samples were determined by qPCR using 2.5% of the precipitated sample DNA and 0.02% of the input DNA. PCR primers used for ChIP are available upon request. Briefly, cells were cross-linked with 1% formaldehyde for 10 min and incubated for 5 min at room temperature with 0.125 M glycine to terminate the reaction. Nuclei were prepared, digested with 50 U micrococcal nuclease for 15 min at 37° C, and sonicated to yield chromatin fragments (200-400 bp). The precleared chromatin was incubated overnight at 4° C with H3K4me3 (Millipore, 07-473), H3K27me2 (Millipore, 07-452), H3K9me3 (Abcam, Ab8898), followed by incubation with protein A agarose (Millipore), which was pre-equilibrated with sonicated salmon sperm DNA and bovine serum albumin. Immunoprecipitated material was washed extensively, and the crosslinks were reversed. DNA from the eluted chromatin was purified by phenol extraction and ethanol precipitation.

5. Drug treatment and siRNA transfection

5-Aza-2'-deoxycytidine (5-Aza-CdR) was obtained from Sigma. Cells were seeded 24 h before treatment and drugs were administered as noted in the figure legends. Predesigned siRNAs specific for *LPHN2* were purchased from M-biotech (Hanam, Korea) and control siRNAs with scrambled sequences were obtained from Qiagen. To confirm whether *LPHN2* expression affects chemosensitivity, si*LPHN2* was transfected into DKO, SNU484, and SW480 cells for 8 h using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Cells were subsequently plated into 96-well plates and cell viability assays were performed in the presence of cisplatin.

6. Cell growth inhibition assays

Tetrazolium dye (MTT; Sigma-Aldrich) assays were used to evaluate the inhibitory effect of cisplatin or 5-Aza-CdR on cell growth. Cells were seeded in 96-well plates, incubated for 24 h, and treated with cisplatin alone or in combination with 500 nM 5-Aza-CdR for 72 h at 37° C. After drug treatment, MTT solution was added to each well and cells were incubated for 4 h at 37° C before removal of the media. Cells were then treated with DMSO (Sigma) and the absorbance of the converted dye in living cells was measured using a microplate reader (Versa-Max, Molecular Devices) at a wavelength of 540 nm. Six replicate wells were used for each analysis and three independent experiments were performed.

Table 1. Primer sequences for RT-PCR and Quantitative real-time PCR

Gene	Sequence (5' ->3')		Size
LPHN2	F	TACCTCACACTGGGTGAATCAG	222bp
	R	GCCTTGTTATAACTCCGTCCAG	

Table 2. Primer sequences for bisulfite sequencing and pyrosequencing

Gene		Sequence (5' ->3')		Size
LPHN2_BS_reg1	F	ATATAGTTAATAAAGTTAAAGTTTAGTTGG	Bisulfite sequencing	353bp
	R	TAAACCCAAAAACTTACTCCCAACCT		
LPHN2_BS_reg2	F	GGAAAATGTTGATTTAATTTTAGGAGT	Bisulfite sequencing	320bp
	R	CAAATCTCCRAAACTAAACACAAAAAC		
LPHN2_1_pyro	F	TTTGGTTAGGTGAGTGGAGAAAAGAG	Pyrosequencing	110bp
	R	[5BIOTIN]CTCCCCTCTAAACCCAAAAACTTACT		
LPHN2_1_seq		CAGAAGGGGTGGGTGA	Pyrosequencing	
LPHN2_pyro2	F	GGGAGTAAGTTTTTGGGTTTAGAGG	Pyrosequencing	220bp
	R	[5BIOTIN]CCCCAAACTCCACTCACTAC		
LPHN2_pyro2_seq		GTGGATAGGATGGAGA	Pyrosequencing	

RESULTS

1. Epigenetic silencing of *LPHN2* in human gastric and colorectal cancer cells

To identify novel genes silenced by DNA methylation, I compared the expression profile of HCT116 cells with that of DKO cells, which are *DNMT1*/*DNMT3B* double knockout HCT116 cells [23]. By comparing microarray expression data sets, I found 1,164 genes (log2 fold change >2) whose expression was responsive to *DNMT1*/*DNMT3B* double knockout (Fig. 1). Among 1,164 differentially-expressed genes, 782 genes, including latrophilin (*LPHN*), a neuronal adhesion G-protein-coupled receptor (GPCR) [26], were up-regulated in DKO cells compared to control HCT116 cells (Fig. 1). Interestingly, I found that *LPHN2* was silenced in six gastric and 13 colorectal cancer cell lines, respectively (Fig. 2A and 2B). *LPHN2* has a CpG island located in the promoter and exon 1 region (Fig. 2A), therefore I investigated the question of whether DNA methylation is responsible for transcriptional silencing of *LPHN2*. In bisulfite sequencing analyses of the two CpG-rich regions within the *LPHN2* CpG island, I found that SNU601 gastric cancer cells and HCT116, LOVO colon cancer cells, which show low *LPHN2* expression, have heavily methylated CpG sites (Fig. 2C). In contrast, SNU484 gastric cancer cells and DKO and SW480, two

colon cancer cells that express high levels of *LPHN2*, have no DNA methylation in the same region (Fig. 2C).

Histone modifications cooperate with DNA methylation in regulation of gene expression [27]. Therefore, I investigated the question of whether the transcriptional silencing observed in SNU601 and HCT116 cells was associated with histone modifications. In our chromatin immunoprecipitation (ChIP) experiments, consistent with *LPHN2* mRNA expression levels (Fig. 2B), SNU601 and HCT116 cells were enriched for repressive histone marks, H3K27 di-methylation (H3K27me2) or H3K9 tri-methylation (H3K9me3), with low levels of active histone marks, H3K4 tri-methylation (H3K4me3) (black line; Fig. 2D). In addition, results of the ChIP assay from SNU484 and DKO cells showing high *LPHN2* expression showed the opposite tendency compared to SNU601 and HCT116 cells (grey line; Fig. 2D). Taken together, these data suggest that widespread transcriptional silencing of *LPHN2* may be caused by epigenetic alterations in human gastric and colon cancer cells.

Figure 1.

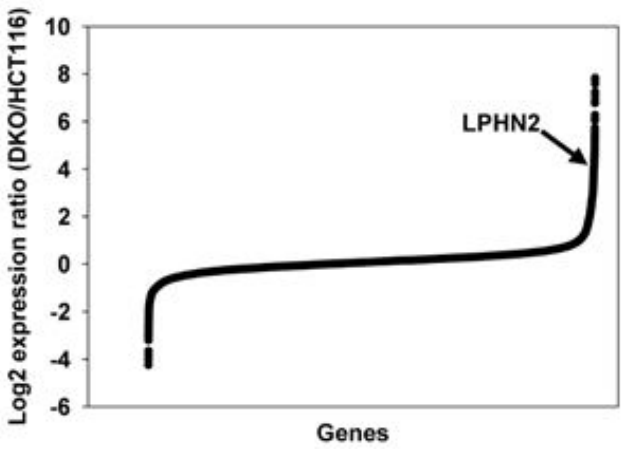

Figure 1. Microarray analyses of mRNA expression in HCT116 and DKO cells

Microarray analyses were performed in HCT116 cells and *DNMT1*/*DNMT3B* double knockout derivative DKO cells. Microarray data results are shown as the log2 expression ratio of genes between HCT116 and DKO. The black arrow indicates *LPHN2*.

Figure 2.A

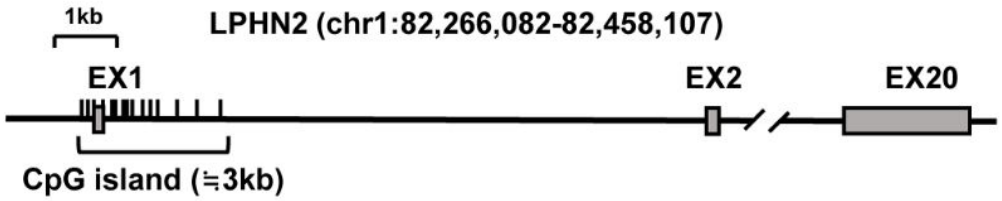

Figure 2.B

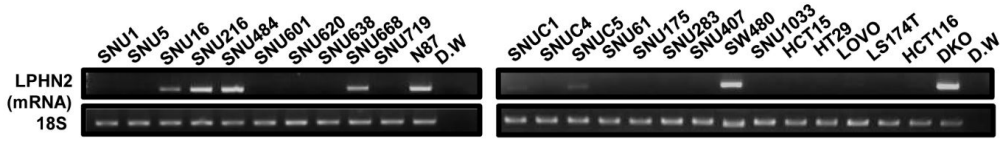

Figure 2. DNA methylation and histone modifications of *LPHN2* in human gastric and colon cancer cells

(A) Schematic representation of *LPHN2*. *LPHN2* exons are indicated by gray boxes. Vertical bars represent each CpG site. The CpG island is indicated according to UCSC genome browser. (B) *LPHN2* mRNA from 11 gastric cancer cell lines (left column) and 15 colorectal cancer cell lines (right column) was analyzed by RT-PCR. 18S ribosomal RNA served as an internal control.

(Figure 2, continued)

Figure 2.C

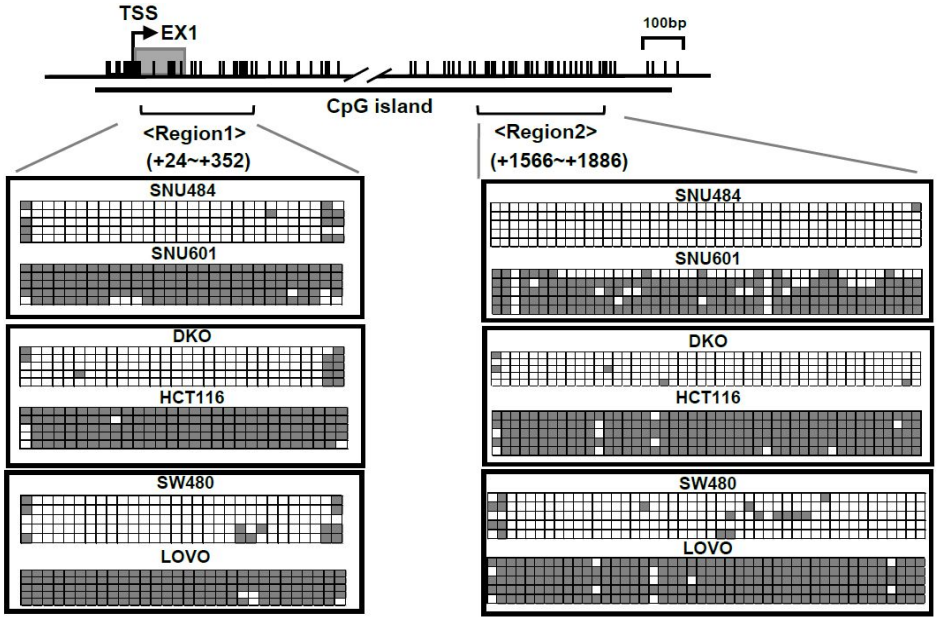

Figure 2.D

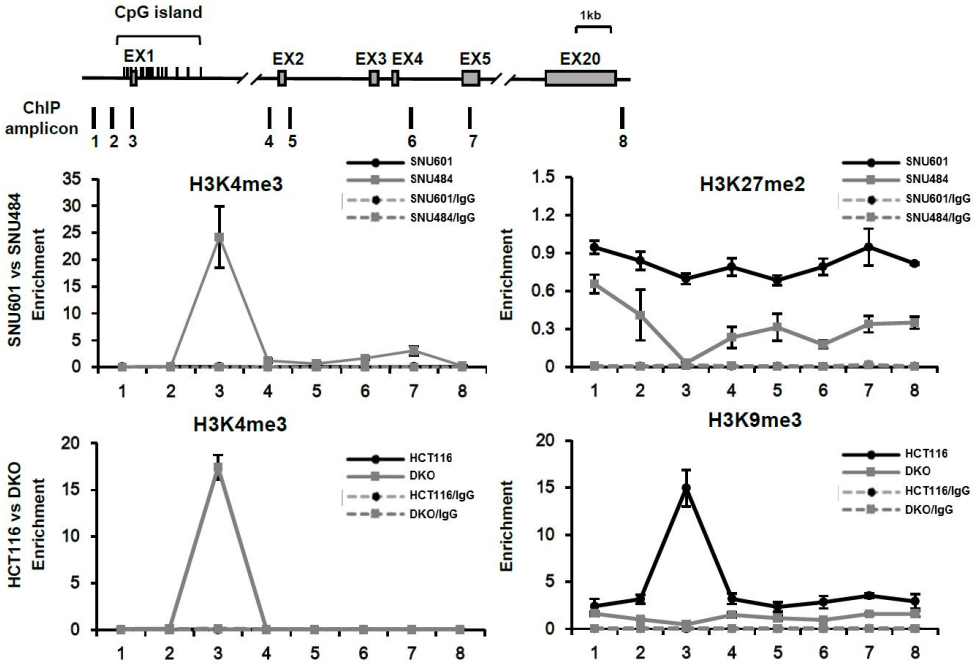

Figure 2. DNA methylation and histone modifications of *LPHN2* in human gastric and colon cancer cells

(C) Bisulfite genomic sequencing analyses of the *LPHN2* CpG islands in SNU601, SNU484, HCT116, DKO, LOVO, and SW480 cells. Each row of squares denotes a single plasmid cloned and sequenced from PCR products amplified from bisulfite-treated gDNA. Open and filled squares represent unmethylated and methylated CpG sites, respectively. *TSS*, transcriptional start site. (D) Characterization of chromatin modification patterns around *LPHN2*. ChIP assays were performed in SNU601, SNU484, HCT116, and DKO cells using antibodies specific for the active histone marker H3K4me3 and the inactive histone markers H3K27me2 and H3K9me3. Enrichment was measured using qRT-PCR. Error bars represent the standard error of the mean (SEM) for triplicate chromatin preparations.

2. Demethylation of the *LPHN2* CpG island restores *LPHN2* expression

To determine whether transcriptional silencing of *LPHN2* was induced by *de novo* CpG methylation of the *LPHN2* promoter region, cells were treated with the demethylating agent 5-Aza-2' -deoxycytidine (5-Aza-CdR). *LPHN2* expression was restored in a dose- and time-dependent manner after treatment of SNU601 and HCT116 cells with 5-Aza-CdR (Fig. 3A-B).

To confirm that reactivation of *LPHN2* was the result of demethylation of *LPHN2* CpG islands, the methylation status of the *LPHN2* promoter region was analyzed using pyrosequencing analyses after treatment of SNU601 and HCT116 cells with 5-Aza-CdR. As shown in Figure 3C, treatment with 500 nM 5-Aza-CdR reduced DNA methylation at the *LPHN2* CpG islands in SNU601 and HCT116 cells (Fig. 3C).

Next, because histone modifications were also involved in epigenetic regulation of gene expression[28], I hypothesized that treatment with 5-Aza-CdR could dynamically affect histone modifications. Consistent with transcriptional reactivation and demethylation, results of ChIP assays showed enrichment of active histone markers (H3K4me3) near the *LPHN2* coding region in SNU601 cells after 5-Aza-CdR treatment (Fig. 3D; left). Interestingly, inactive histone marks (H3K27me2) were also significantly enriched after treatment with 5-Aza-CdR (Fig. 3D; right), supporting the previous finding that promoter regions of

methylated genes are frequently enriched by bivalent histone modifications in human cancer cells [29].

In summary, *LPHN2* mRNA expression was restored after 5-Aza-CdR treatment, followed by demethylation of the heavily methylated CpG island of the *LPHN2* promoter region. In addition, treatment with 5-Aza-CdR can dynamically affect histone modifications near the *LPHN2* coding region.

Figure 3.A

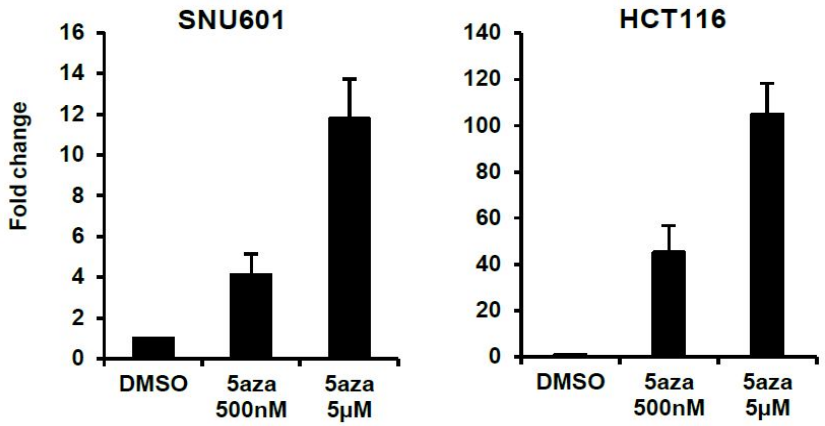

Figure 3.B

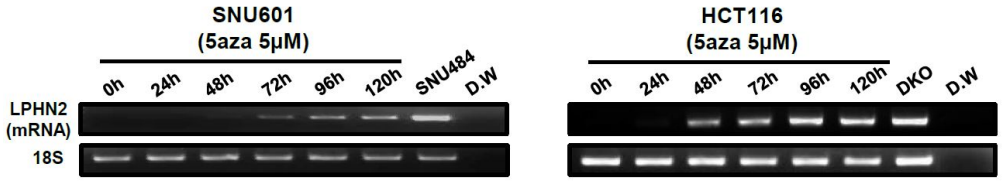

Figure 3. Restoration of *LPHN2* expression after treatment with 5-Aza-CdR

(A) SNU601 and HCT116 cells were treated with DMSO or 5-Aza-CdR for 4 days. *LPHN2* mRNA expression was measured using qRT-PCR. Each value was normalized to 18S ribosomal RNA. Error bars represent the standard deviation (S.D.) for three independent RNA preparations. (B) SNU601 and HCT116 cells were treated with 5 μM 5-Aza-CdR for 5 days. *LPHN2* mRNA and 18S ribosomal RNA expression was analyzed by RT-PCR at the indicated times.

(Figure 3, continued)

Figure 3.C

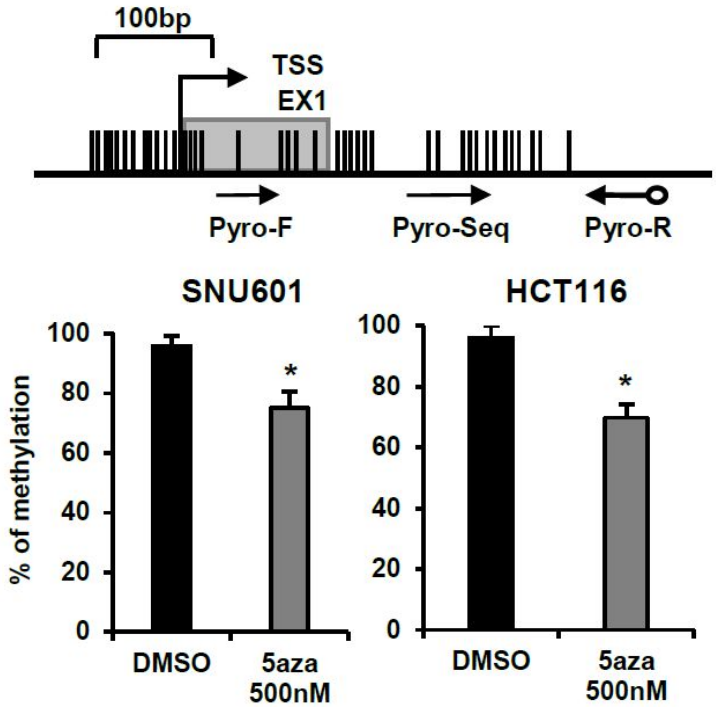

Figure 3.D

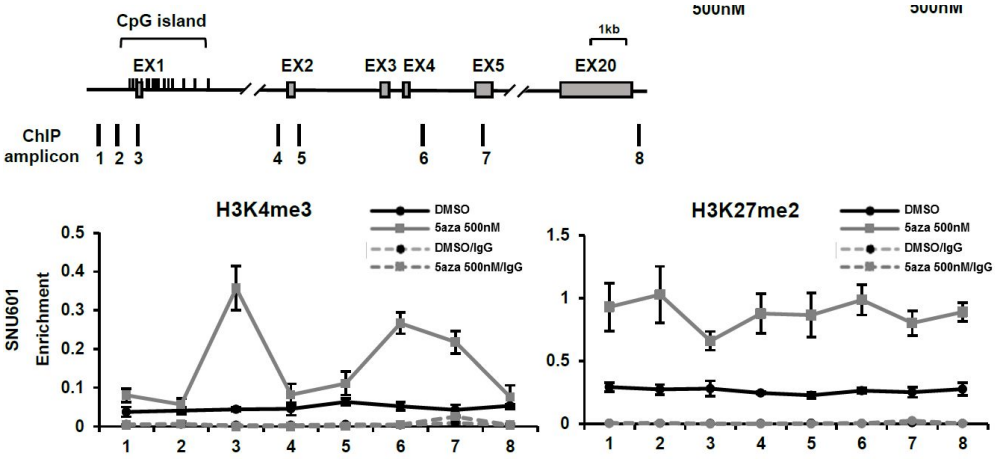

Figure 3. Restoration of *LPHN2* expression after treatment with 5-Aza-CdR

(C) After treatment with DMSO or 500 nM 5-Aza-CdR, the quantitative methylation status of *LPHN2* in SNU601 and HCT116 cells was determined by pyrosequencing. The location of analyzed CpG sites corresponding to the pyrosequencing primer set is indicated by arrows. Error bars represent the S.D. for three independent preparations of bisulfite-treated gDNA. *indicates P <0.05 using Student's t-tests. (D) Dynamics of histone modifications after 5-Aza-CdR treatment in SNU601 cells. Chromatin was prepared from SNU601 cells treated with DMSO or 5-Aza-CdR (500 nM) for 4 days. Enrichment of the active histone mark (H3K4me3) and inactive histone mark (H3K27me2) was measured using qRT-PCR. Data are presented as the mean ± SEM for three independent chromatin preparations.

3. Methylation status and *LPHN2* mRNA levels in primary gastric and colon tumor tissues

To determine whether DNA methylation-mediated transcriptional silencing of *LPHN2* also occurs in human primary gastric tissue, analysis of *LPHN2* mRNA expression and methylation status was performed using qRT-PCR and pyrosequencing. Compared with their normal counterparts, 18 of 24 (75%) primary gastric tissues showed low endogenous levels of *LPHN2* mRNA (Fig. 4A).

Among these 18 primary gastric tissues, 9 of 18 (50%) tissues have hypermethylated *LPHN2* CpG islands (Fig. 4B). *LPHN2* methylation in primary human gastric cancer tissues was also confirmed (Fig. 4C and 4D). These results indicate that transcription of *LPHN2* is regulated by DNA methylation in human gastric cancer tissues. Similar results were obtained with human colon tumor tissues (Fig. 4E and data not shown).

Figure 4.A

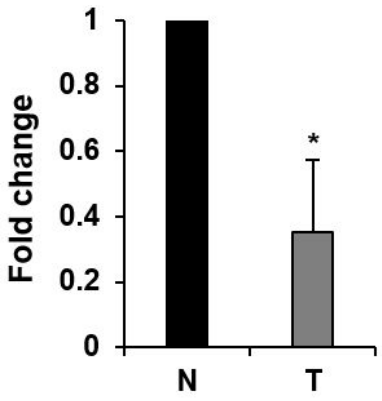

Figure 4.B

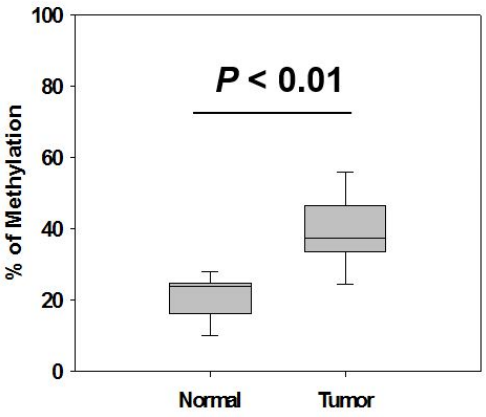

Figure 4. Loss of *LPHN2* expression in human gastric and colon primary tissues

mRNA expression in primary gastric tissues was analyzed using qRT-PCR and the methylation status of *LPHN2* was confirmed via pyrosequencing analyses. Loss of *LPHN2* mRNA expression was shown in 18 of 24 primary gastric tissues. (A) A summary of *LPHN2* expression in 18 primary gastric tissues compared with normal tissues presented as fold change of relative mRNA expression. (B) Among 18 primary gastric tissues, 9 of 18 showed hypermethylation of *LPHN2* CpG islands compared to normal control tissues. The methylation ratio percentage is also presented as a box plot.

(Figure 4, continued)

Figure 4.C Figure 4.D Figure 4.E

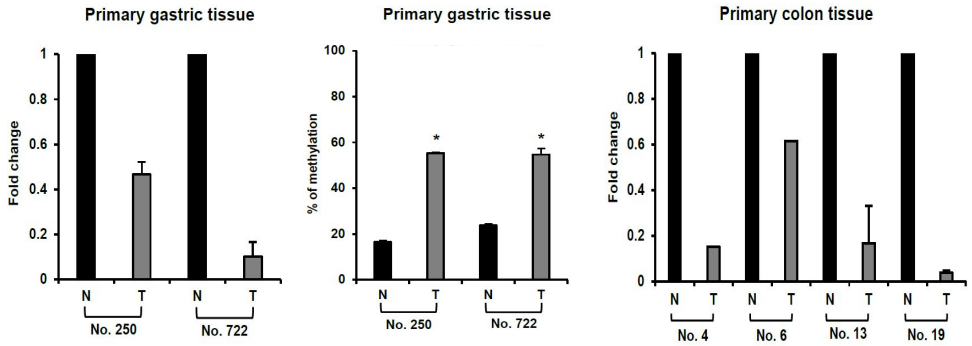

Figure 4. Loss of *LPHN2* expression in human gastric and colon primary tissues

(C) *LPHN2* expression levels in primary gastric tissues were analyzed using qRT-PCR. *LPHN2* expression was normalized to 18S ribosomal RNA and is shown as fold change relative to their normal tissue samples. Data are expressed as the mean ± S.D. (D) Bisulfite-treated gDNAs from patient No. 250 and No. 722 were used to determine the methylation status of the *LPHN2* promoter region using pyrosequencing analyses. (E) *LPHN2* expression levels in primary colon tissues were analyzed by qRT-PCR and are shown as fold change relative to their normal tissue samples. *N*, normal tissue; *T*, tumor tissue. * indicates $P < 0.01$ using Student's t-test.

4. Cancer cells with *LPHN2* methylation exhibit higher sensitivity to cisplatin

Next I examined the question of whether *LPHN2* methylation status could influence the chemosensitivity of cancer cells. First, HCT116 and DKO cells were exposed to increasing concentrations of cisplatin and cell viability was measured after 72 h. Interestingly, DKO cells without *LPHN2* methylation were more resistant to cisplatin than their parental HCT116 cells, with methylated *LPHN2* (left column, Fig. 5A). The *LPHN2* methylation status and drug sensitivity to cisplatin were also compared in two gastric cancer cell lines (SNU601 and SNU484) and two colon cancer cell lines (LOVO and SW480). SNU601 and LOVO cells show a *LPHN2* methylation pattern similar to that of HCT116 cells, whereas SNU484 and SW480 cells show a methylation pattern similar to that of DKO cells (Fig.2). As expected, our results in SNU601 and LOVO cells were similar to results in HCT116 cells, and those in SNU484 and SW480 were similar to the results obtained in DKO cells (middle and right column, Fig. 5A).

Next, I investigated the combined effect of cisplatin and 5-Aza-CdR on the viability of HCT116, SNU601, and LOVO cells with methylated *LPHN2*. Treatment of cells with this drug combination had an attenuated inhibitory effect on cell growth compared with cisplatin only (Fig. 5B), suggesting that the

methylation status of the genome may influence the chemosensitivity of cancer cells to cisplatin.

To further examine whether *LPHN2* expression is responsible for the inhibitory effect of cisplatin on cell growth, knock down of *LPHN2* expression was performed using *LPHN2*-directed siRNA (Fig. 5C). Cell viability was assessed after treatment with cisplatin for 72 h (Fig. 5D). As shown in Figure 5D, reduction of *LPHN2* expression enhanced the anti-proliferative effects of cisplatin in DKO, SNU484, and SW480 cells. Taken together, these results suggest that the *LPHN2* methylation status shows strong correlation with cancer cell chemosensitivity to cisplatin.

Figure 5.A

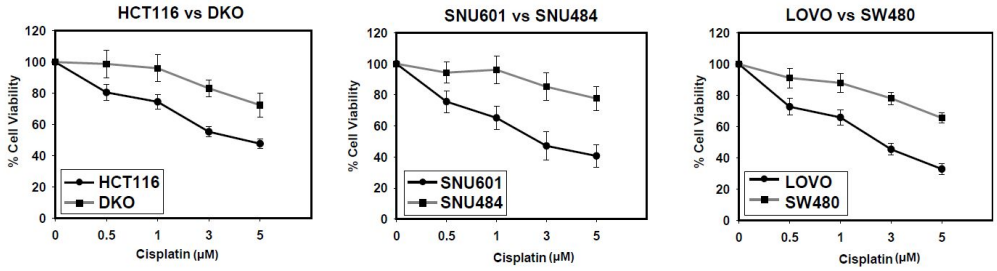

Figure 5.B

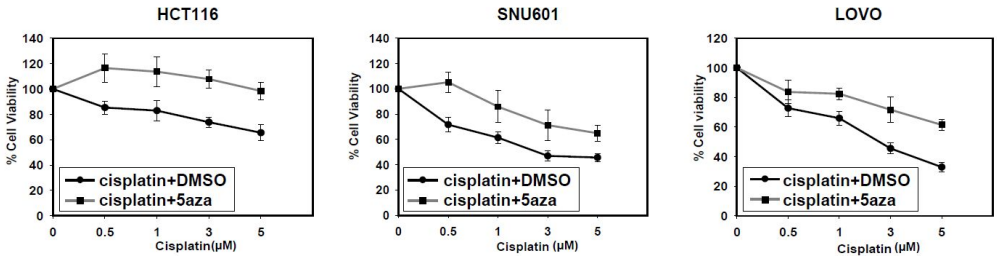

Figure 5. Methylation status of *LPHN2* determines the differential response to cisplatin

(A) Cells were treated with increasing concentrations of cisplatin (0.5, 1, 3, or 5 μM) for 72 h. Cell viability was confirmed using MTT assays. Cell viability is expressed as a percentage relative to untreated cells. Data represent the mean ± S.D. of three independent experiments. (B) Cells were treated with DMSO or 500 nM 5-Aza-CdR in combination with different doses of cisplatin (0.5, 1, 3, or 5 μM). The number of viable cells was measured after 72 h. Data are expressed as the mean ± S.D. (n=3).

(Figure 5, continued)

Figure 5.C

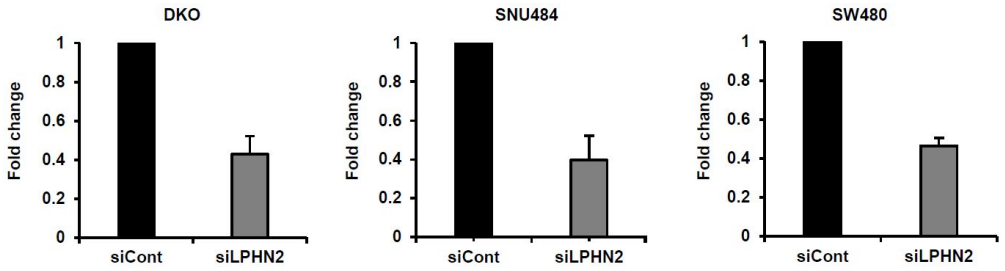

Figure 5.D

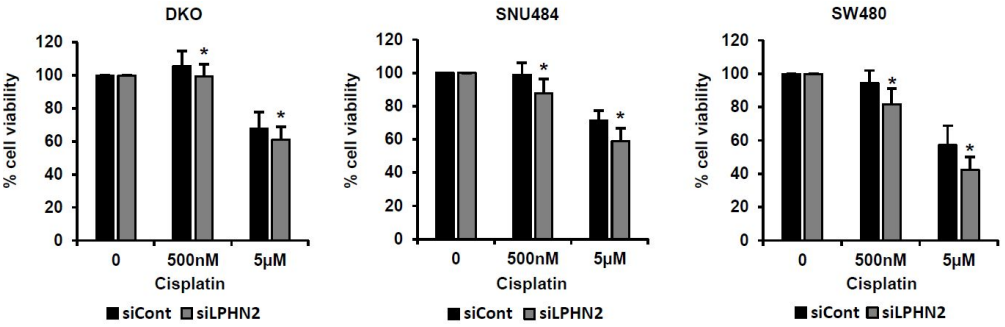

Figure 5. Methylation status of *LPHN2* determines the differential response to cisplatin

(C–D) Cells were transfected with control or *LPHN2*–specific siRNAs for 8 h. Cells were plated in 96–well plates, followed by cisplatin treatment (0.5 or 5 μM) for 72 h. (C) *LPHN2* expression and (D) cell viability were measured using qRT–PCR and MTT assays, respectively. Error bars represent the S.D. (n=3, $*P$ <0.05).

Figure 6.A

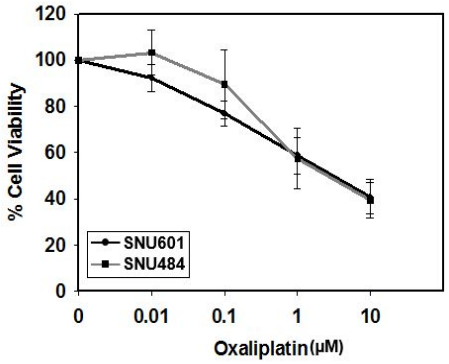

Figure 6.B

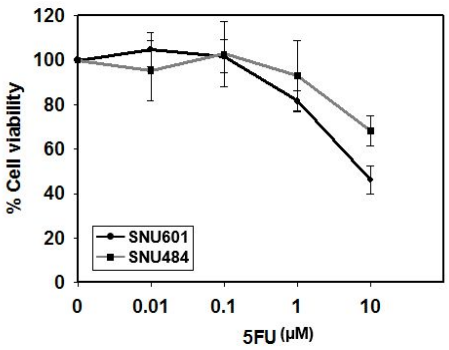

Figure 6.C

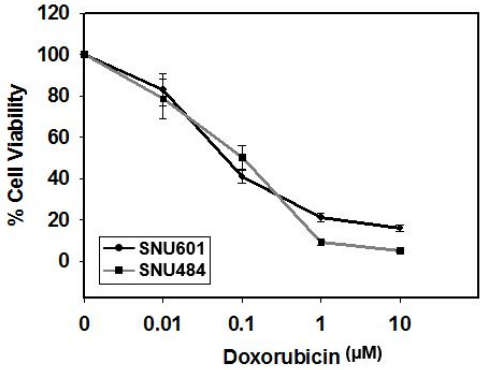

Figure 6.D

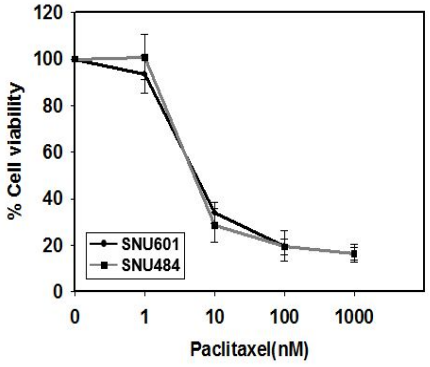

Figure 6. Methylation status of *LPHN2* does not confer differential sensitivity to various chemotherapeutic agents

Cells were treated with the indicated doses of (A) oxaliplatin, (B) 5-FU, (C) doxorubicin, and (D) paclitaxel for 72 h. Cell viability was confirmed using MTT assays. Data are expressed as the mean ± S.D (n=3).

DISCUSSION

LPHN is a neuronal adhesion G-protein-coupled receptor that can stimulate neuronal exocytosis in vertebrates [20,21]. The *LPHN* gene family consists of *LPHN1*, *LPHN2*, and *LPHN3* [26]. Among these, genetic alterations of *LPHN2*, which is believed to be a p53 target gene [30], are frequently observed in various human cancers [31,32]. In the current study, I found DNA methylation-mediated transcriptional silencing of *LPHN2* in human gastric and colon cancer.

By comparing microarray expression data sets, I identified differential expression of *LPHN2* in HCT116 cells and DKO cells, the *DNMT1* and *DNMT3B* double knockout derivative of HCT116 cells (Fig. 1). High *LPHN2* expression in DKO cells, but not HCT116 cells, could be a presumed restoration of silenced genes mediated by the lack of DNMTs in DKO cells [23,33]. Our bisulfite sequencing analyses confirmed that gastrointestinal cancer cells with undetectable *LPHN2* expression have hypermethylated *LPHN2* CpG islands (Fig. 1). Moreover, 5-Aza-CdR treatment induced activation of methylated *LPHN2* and altered histone modifications around the *LPHN2* promoter

(Fig. 2). I also found that *LPHN2* methylation is common in primary human gastric and colon cancer tissues (Fig. 3), suggesting that DNA methylation-mediated transcriptional silencing of *LPHN2* is a cancer-specific epigenetic event.

What is the functional significance underlying the DNA methylation-mediated transcriptional loss of *LPHN2* in human cells? According to recent studies, increasing evidence indicates that the DNA methylation status can influence the anti-proliferative effects of DNA-damaging agents [34,35,36]. Additionally, the cancer cell-specific combination of DNA methylation and repressive histone modifications can be used as a potential target for epigenetic therapies [16]. Interestingly, I found that cancer cells that express high levels of *LPHN2* were more resistant to cisplatin treatment than HCT116, SNU601, and LOVO cells, which have methylated *LPHN2* (Fig. 4). Moreover, combined treatment with cisplatin and 5-Aza-CdR overcame the cytotoxicity of cisplatin in cancer cells with methylated *LPHN2*. siRNA-mediated down-regulation of *LPHN2* expression increased the anti-proliferative effects of cisplatin in cancer cells that expressed high levels of *LPHN2* (Fig. 4C). However, the *LPHN2*

methylation status did not affect differential sensitivity to other chemotherapeutic drugs (Fig. 6). Therefore, our results strongly suggest that the methylation status of *LPHN2* is a potential novel epigenetic biomarker for cisplatin treatment in patients with gastrointestinal cancer.

In conclusion, I identified the molecular mechanism for transcriptional silencing of *LPHN2*. Our data highlight its functional role as an epigenetic biomarker for cisplatin treatment in gastrointestinal cancer. However, further studies are required to evaluate the clinicopathological features and outcomes with respect to cisplatin chemosensitivity and the methylation status of *LPHN2*.

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국문 초록

암은 유전적 변이 또는 후성유전학적 변이에 의해 정상적으로 조절되어야 하는 암유전자와 암억제유전자의 발현에 문제가 생긴 질병으로서 정상적인 세포의 성장/사멸과 분화가 저해되면서 발생하는 질병 중 하나이다. 암화 과정에 기여하는 후성유전학적 변이로는 DNA methylation, histone modification 등이 있으며, 비정상적인 유전자의 발현에 있어 다양한 후성유전학적 변이가 관여한다고 알려져 있다.

최근 연구에 따르면, 특정한 유전자의 후성유전학적 변이가 인간의 암을 진단하는 데 있어 바이오마커로서 사용될 수 있다. 이를 이용함으로써 암의 진단 및 임상응용, 더 나아가 항암제 감수성에 적용하려는 연구 또한 지속해서 진행되고 있다. 그러나 인간의 위장관암에서 항암제의 내수성에 관한 표준화된 후성유전학적 바이오마커가 존재하지 않기 때문에 다음과 같은 연구를 진행하였다.

먼저 대장암 세포주인 HCT116과 DKO의 microarray data를 비교하여 DNA methlyation에 의해서 조절되는 유전자 후보군을 찾았다. DKO는 DNA methyltransferase인 DNMT1과 DNMT3B가 knockout된 세포주로서, 같은 세포주 이지만 유전자의 발현 차이가 나는 것은 DNA methylation에 의한 조절이라고 예측할 수 있다. 후보군 중에서도, LPHN2는 다양한 신호전달 경로에 관여할 수 있는 G-protein coupled receptor이며, 이의 후성유전학적 변이에 대한 보고가 존재하지 않았으므

로 연구를 시작하였다. Bisulfite sequencing 기법과 pyrosequencing 기법으로 LPHN2 유전자가 DNA methylation에 의해서 조절되어 있음을 밝혔고, 그에 따라 ChIP assay를 통해 histone modification 또한 같이 관여하고 있음을 증명하였다. 또한 DNA methyltransferase inhibitor를 처리하였을 때, LPHN2의 발현이 증가 하는 것이 LPHN2의 CpG island에 존재하고 있던 methylated CpG site들이 demethylation되어 발생하는 현상임을 확인하였고, 또 그에 따라 histone modification도 dynamic하게 변화하는 것을 확인하였다. LPHN2가 DNA methylation에 의해서 저해 되어 있는 이러한 현상은 위암과 대장암 세포주뿐만 아니라 환자의 primary tissue에서도 발견되었다.

흥미롭게도, 본 연구에서는 LPHN2의 발현이 높은 세포주에서 DNA-damaging agent인 cisplatin에 더욱 높은 감수성을 갖는다는 것을 발견하였다. 또한 methylation 되어있는 LPHN2를 가진 세포주에 5-Aza-2'-deoxycytidine 와 cisplatin을 조합해서 처리하였을 땐 세포독성이 오히려 더 감소하였음을 확인하였다. 더 나아가 siRNA를 이용하여 LPHN2의 발현을 저해 하였을 때에는 cisplatin의 항암효과에 더욱 더 높은 sensitivity를 보임을 증명하였다. 다른 항암제와는 LPHN2의 methylation level과 그 감수성의 상관관계가 파악되지 않음에 따라 LPHN2의 methylation 상태는 인간의 위암과 대장암에서 cisplatin에 대한 감수성을 결정한다고 할 수 있다.

마지막으로 본 연구에서는 다양한 epigenetic alteration들이 LPHN2의 발현을 저해시켰으며, 이러한 현상이 인간의 위암과 대장암에서 cisplatin에 대한 반응을 강화할 수 있음을 제시하였다. 따라서 LPHN2의 methylation 상태는 위암과 대장암 환자에 cisplatin을 이용한 치료하는 데 있어 이를 새로운 후성유전학적 바이오마커로 사용할 수 있음을 제시하고 있다.

Keywords : 후성유전학, DNA methylation, histone modification, 5-Aza-2'-deoxycytidine, LPHN2, chemosensitivity

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