Dynamic alteration of Dclk1 isoform expression in gastric carcinogenesis

HyeKyeong Hwang

Department of Medical Science

The Graduate School, Yonsei University

Dynamic alteration of Dclk1 isoform expression in gastric carcinogenesis

Directed by Professor Ki Taek Nam

The Master's Thesis
Submitted to the Department of Medical Science,
the Graduate School of Yonsei University
in partial fulfillment of the requirements for the degree of
Master of Medical Science

HyeKyeong Hwang

June 2015

This certifies that the Master's Thesis of
HyeKyeong Hwang is approved.

Thesis Supervisor: Ki Taek Nam

Thesis committee Member #1: Yong Chan Lee

Thesis committee Member #2: Jun Young Seo

The Graduate School
Yonsei University

June 2015

ACKNOWLEDGEMENTS

I would like to express my gratitude to my advisor Professor Ki Taek Nam. I thank him for introducing me to the subject as well for sharing expertise and valuable guidance and engagement through the learning process of this master thesis. His passion and enthusiasm for research has raised my love of science and this is what inspired me to be productive during my graduate experience.

I also would like to thank Professor Yong Chan Lee and Professor Jun Young Seo for their time and inspirational discussions regarding this study. Their variety perspectives have helped me strengthen my work.

My sincere thank goes to Dr. Yu for helping me in every step. I very much appreciate her contributions of time, ideas, encouragement, and enormous amount of help throughout the entire process.

I thank my colleagues for their support and being a source of energy in the past two years.

Lastly, I would like to thank my family and friends for their deep support, love and encouragement.

HyeKyeong Hwang

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLE

<ABSTRACT>

Dynamic alteration of Dclk1 isoform expression in gastric carcinogenesis

HyeKyeong Hwang

Department of Medical Science

The Graduate School, Yonsei University

(Directed by Professor Ki Taek Nam)

Dclk1 was proposed as a putative quiescent stem cell marker in the intestine, but also identified as a marker of tuft cells in the stomach. Dclk1 has multiple transcript variants that encode different isoforms, long form (LF, full-length), short form (SF, kinase domain only), and 363 isoform (doublecortin domain only). Prior studies have noted the expression of Dclk1 in association with various cancers. However, no investigations have studied the expression of Dclk1 isoforms in association with metaplasia and gastric cancer. Here, using DMP-777 treated mouse SPEM model, altered expression of Dclk1 isoforms in association with gastric carcinogenesis was analyzed by western blotting and qPCR analysis. Both LF and SF of Dclk1 expression intensity increased in all regions of the gastric mucosa as metaplasia was induced. In contrast to translational level, gene expression patterns of each isoforms varied regionally. In addition, immunohistochemistry and *in situ* hybridization(ISH) analyses confirmed different distribution of Dclk1 isoforms as well as altered expression of Dclk1 isoforms involved in the course of gastric carcinogenesis. Especially, strong SF-expressing cells appeared in the neck-isthmus region as metaplasia was induced compared to normal gastric mucosa. Moreover, examination of Dclk1 isoforms in resected specimens of gastric cancer patients revealed loss of LF in cancer while it existed in normal and intestinal metaplasia (IM). These results provide possibility of Dclk1 LF as a new cellular and molecular marker in gastric cancer progression.

Key words: Dclk1, gastric carcinogenesis, SPEM, DMP-777

Dynamic alteration of Dclk1 isoform expression in gastric carcinogenesis

HyeKyeong Hwang

Department of Medical Science
The Graduate School, Yonsei University

(Directed by Professor Ki Taek Nam)

I. INTRODUCTION

Gastric cancer develops through a multiple stages that include chronic gastritis, oxyntic atrophy, foveolar hyperplasia, and metaplasia.[1] Metaplasia by loss of parietal cells (oxyntic atrophy) in fundus of the stomach is often highly correlated with development of gastric cancer.[2,3] These acid producing parietal cells are the key player in HCl secretion. In addition, it also secretes other regulators such as sonic hedgehog, transforming growth factor (TGF)-α, amphiregulin, and heparin binding-epidermal growth factor (HB-EGF).[4,5] Also, several studies have found that parietal cells affect the differentiation of mucous neck cells into chief cells, indicating that absence of parietal cell can alter the gastric mucosa leading to metaplasia.[6]

Two types of metaplasia develop in precursor to gastric cancer: spasmolytic polypeptide-expressing metaplasia (SPEM) and intestinal metaplasia.[2] SPEM derives from transdifferntiation of mature chief cells and is only restricted to fundus and express trefoil factor 2 (TFF2).[2,7] Several mouse models are available to induce SPEM including *Helicobacter felis*, DMP-777, and L-635. Chronic infection with *Helicobacter felis* for about 6 months leads to loss of parietal cell and emergence of SPEM with inflammation.[4,7] Oral administration of L-635 results in inflammation and induces SPEM by 3 days of treatment.[2] DMP-777 is a neutrophil elastase inhibitor, but it also acts as a parietal cell specific protonophore thereby selectively ablating parietal cells. Oral dose of DMP-777 treatment for 14 days leads to emergence of SPEM without inflammation. Moreover, these changes are reversible when DMP-777 is suspended after treatment.[2,5,7] Another type of metaplasia is intestinal metaplasia

(IM) which develops in both fundus and antrum and is characterized by intestinal goblet cells.[2]

Doublecortin-like kinase 1 (Dclk1) is a microtubule associated protein kinase and has been known to be involved in development of nervous system.[8,9] However, emerging investigations have proposed its roles as a quiescent intestinal stem cell marker.[10,11] The expression of Dclk1 was first identified in the isthmus of gastric glands and +4 position of intestinal crypts where stem cells are thought to reside.[10] It was also reported that Dclk1 positive cells did not incorporate BrdU, and did not colocalize with other markers of differentiated gastric cells, suggesting that these are putative gastrointestinal stem cell marker.[10] These findings were further confirmed by May and coworkers through immunohistochemcial staining in +4 position of stem cell zone within the small intestine and colonic crypts of mouse.[11]

Challenging arguments against Dclk1 as a gastrointestinal stem cell marker have been raised when Gerbe and colleagues found majority of the Dclk1-expressing cells in the villi.[12] They found these cells expressed cyclooxygenase (COX) 1 and 2 as well as high levels of tubulin and villin, which are characteristics of tuft cells.[12] Tuft cells, also known as brush or calveolated cells, are characterized by their stiff microvilli in apical and a unique tubulovesicular system.[13] These cells constitute the unusual type of epithelial cells in several organs of respiratory and gastrointestinal tract.[13,14] Tuft cells express markers related to chemoreception such as the guanine nucleotide binding protein α-transducing 3 (GNAT3), α-gustducin, the transient receptor potential cation channel, subfamily M, member 5 (TRPM5), and the G-protein coupled taste receptor type 1 member 3 (TAS1R3). [14-16] These findings provide an evidence of Dclk1 as a tuft cell marker.[12]

Recently, Nakanishi and colleagues have revealed that Dclk1 is rather a marker for intestinal cancer stem cells and it was further suggested that targeting the Dclk1-positive tumor cells could be a therapeutic potential for colorectal cancer.[17]

Dclk1 contains two doublecortin domains in the N-terminus, kinase domain in the C-terminus, and a proline-rich domain in between doublecortin and kinase domain.[8,18] Through alternative splicing, three main types of transcript splice forms are generated including long form (LF) that contains all the domains, short form (SF) with only the kinase domain, and 363 form with only the doublecortin

domain.[8,18]

Several studies have confirmed the increase expression of Dclk1 in association with cancer.[19-21] High number of Dclk1-expressing cells was reported in the stomach of smad3-null mouse.[22] Bailey et al. have examined the expression of Dclk1 in preinvasive pancreatic neoplasia and found that these cells had distinct morphologies as well as cancer stem cell like properties.[23] Also, recent studies have reported an increased number of Dclk1-expressing cells in association with parietal cell loss and SPEM.[16]

To date, studies on the expression of Dclk1 are well characterized in the small intestine, colon, and pancreas. However, not much research has been done in gastric mucosa. In addition, despite the multiple isoforms, no studies have investigated the relation between the Dclk1 isoforms and gastric carcinogenesis. In this report, the expression of Dclk1 isoforms in association with gastric carcinogenesis was investigated by DMP-777 treated mouse SPEM model. Translational, transcriptional level as well as cellular localization of Dclk1-positive cells was analyzed depending on the regions of the stomach. Moreover, the altered expressions of Dclk1 isoforms were examined in gastric cancer patients.

II. MATERIALS AND METHODS

1. Mice

All experiments were approved by the Institutional Animal Care and Use Committee of Yonsei University College of Medicine. C57BL/6 mice (Orient Laboratories, Seoul, Korea) were purchased at 6 weeks of age and used for all experiments. All mice were given a standard pellet chow diet (PicoLab 5053, LabDiet) *ad libitum* and were maintained in specific pathogen-free conditions.

2. DMP-777 treatment

Preparation and treatment of DMP-777 was performed as previously described.[2,5,24] DMP-777 was a gift from DuPont Pharmaceuticals. DMP-777 was formulated at a concentration of 2% as a suspension in 0.5% methycellulose (Sigma-Aldrich, St. Louis, MO, USA). C57BL/6 mice (6 weeks of age) were administered DMP-777 orally as a gavage (350 mg/kg) once daily for 3, 7, 14, and 28 days.

3. Western blotting

Mice were sacrificed by asphyxiation in a CO_2 chamber. Stomach was excised and opened along the greater curvature and washed with PBS. Twelve different regions within the stomach were punctured and chopped into smaller pieces with sterile scissor/pestle. Tissues were lysed in lysis buffer (20mM HEPES (pH 7.0), 0.15M NaCl, 10% Glycerol, 1% Triton X-100, 1mM EDTA, 1mM EGTA, 10mM β-phosphoglycerate, 1mM Na_3VO_4, 5mM NaF, and protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific, Rockford, IL, USA), and lysates were collected by centrifugation. After determining the concentration of proteins with BCA protein assay (Pierce Biotechnology, Rockford, IL, USA), lysates were mixed with 4X sample buffer and boiled at 95°C for 5 min. 15 μg of total proteins was separated on 10% SDS-PAGE gel and transferred to a PVDF membrane (Millipore, Billerica, MA, USA). After blocking with 5% skim milk for 30 min, membrane was probed with primary antibody at 4°C overnight. Then, HRP-conjugated secondary antibody (Life Technology, Carlsbad, CA, USA) was incubated at room temperature for 1 hr. The proteins were

detected using ECL reagents (Pierce Biotechnology, Rockford, IL, USA), and intensity quantification was measured using Image J (developed by Wayne Rasband). The antibodies used were anti-Dclk1 C-term (Novus Biologicals, Littleton, CO, USA), anti-Dclk1 N-term (Abcam, Cambridge, UK), anti-acetylated tubulin (Sigma-Aldrich, St. Louis, MO, USA), and anti-Gapdh (Abcam, Cambridge, UK).

4. RNA extraction and Quantitative PCR (qPCR)

RNA was extracted from tissues using TRIzol (Life Technology, Carlsbad, CA, USA). Then, 1 μg of DNase treated RNA was used to synthesize cDNAs using the ImProm-II™ reverse transcription system (Promega, Madison, WI, USA). Samples were analyzed in duplicate using POWER SYBR Green Master Mix (Applied Biosystems, Waltham, MA, USA) on a Step I Real-Time PCR System (Applied Biosystems, Waltham, MA, USA). Primer sequences are given in Table 1.

Table 1. Primers used for qPCR and preparation of *in situ* hybridization probe

Genes	Sequences
LF-Fw	TGCCTCCACCAGCTCAGTTAATGGAAC
LF-Rv	TGGATGAAAGTGAAGTCGAGGAGCC
SF-Fw	TAGCTAATCCAACTGCTTCCCA
SF-Rv	TTCTATGAGCTCTAACATGGACAC
363-Fw	TCAGTAGGAGACTCAGTGTAA
363-Rv	TTCAAAGAATACCGCGAGTAG
GAPDH-Fw	CATGGCCTTCCGTGTTCCTA
GAPDH-Rv	CCTGCTTCACCACCTTCTTGAT
ISH-DC-Fw	GGAGATCGATACTTCAAAGGG
ISH-DC-Rv	TCTGATGATGGTGACCAGCTT
ISH-kinase-Fw	ATCCTTAAACTCATCCAACCGA
ISH-kinase-Fw	GTTCTAACATGGACACAGTCTT

5. Immunohistochemistry (IHC)

IHC was followed by previous report[22] with minor modification. Briefly, slides were deparaffinized and rehydrated through 100%, 95% and 70% ethanol. Antigen retrieval was performed using a pressure cooker. Endogenous peroxidase activity was blocked by incubation with 3% H_2O_2 for

30 min then incubated with protein blocking solution (Dako, Glostrup, Denmark) for 1 hr at room temperature. Primary antibody was incubated in a humid chamber at 4°C overnight. Then slides were incubated with secondary rabbit IgG (Dako, Glostrup, Denmark) for 15 min at room temperature, and developed with Dako Envision^{+} System-HRP DAB (Dako, Glostrup, Denmark.). Counterstained with Mayer's Hematoxylin (Sigm-Aldrich, St. Louis, MO, USA) and mounted with mounting solution (Electron Microscopy Sciences, Hatfield, PA, USA). For immunofluorescence, Alexa 568 conjugated anti-rabbit secondary antibody (Life Technology, Carlsbad, CA, USA) was used to detect the binding of anti-Dclk1 C-term. For dual staining of anti-Dclk1 N-term, TSA (Tyramide Signal Amplification)-Plus Fluorescein Kits (PerkinElmer, Waltham, MA, USA) was used to amplify the binding of primary antibody with gastric tissue. Nuclei were stained with DAPI (Vector Laboratories, Inc., Burlingame, CA, USA).

6. *In situ* hybridization (ISH)

In situ hybridization was performed as previously reported[25] with modification. Briefly, 4-μm sections from paraformaldehyde-fixed and paraffin-embedded tissue samples were hybridized with DIG-labeled and/or fluorescein-labeled sense and antisense cRNA probes. Hybridization was performed overnight at 42°C in 2× SSC containing 50% formamide. Tissue sections were then incubated overnight at 4°C with an anti-DIG rhodamine antibody (Roche Diagnostics, Indianapolis, IN, USA). Counterstained with nuclear fast red (Vector Laboratories, Inc., Burlingame, CA, USA). Nuclei were stained with DAPI (Vector Laboratories, Inc., Burlingame, CA, USA).

III. RESULTS

1. Dclk1-expressing cells are increased in metaplasia associated with parietal cell loss

In order to characterize the expression of Dclk1 in association with gastric carcinogenesis, SPEM was induced by DMP-777, which causes parietal cell loss and thereby leads to emergence of SPEM without inflammation. After DMP-777 treatment for 3 days, 7 days, 14 days, and cessation after 14 days, immunohistochemical analysis was performed to examine the expression of Dclk1 (Fig. 1). In normal gastric fundic mucosa, few Dclk1-positive cells were observed in the isthmus region. As DMP-777 was treated and SPEM was induced, increased number of Dclk1-positive cells was observed. Significant increase was detected by 7 days of DMP-777 treatment, and a four-fold increase was observed by 14 days of DMP-777 treatment. Cessation of DMP-777 after 14 days of treatment led to reacquisition of parietal cell as well as regaining of its normal gastric mucosa. In this group, the number of Dclk1-postivie cells was recovered back to the normal level. Few Dclk1-positive cells were also observed in untreated antrum. However, unlike in fundic mucosa, no differences in the number of Dclk1-expressing cells were detected in association with emergence of SPEM. These results suggest that Dclk1-expressing cells increase in metaplasia associated with parietal cell loss.

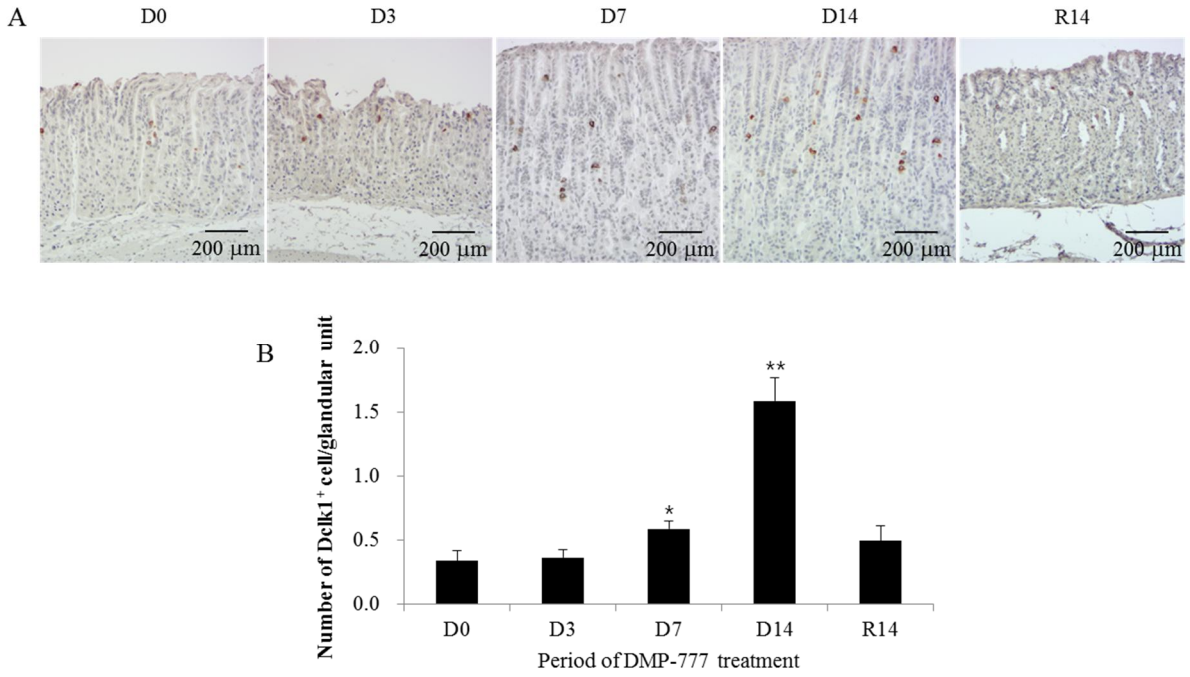

Figure 1. Parietal cell loss by DMP-777 treatment increased the number of Dclk1-positive cells. (A) Immunohistochemical analysis of Dclk1-positive cells in the stomach of DMP-777 treated mice (B) Dclk1-positive cells significantly increased by 7 days and 14 days of DMP-777 treatment. *, $p<0.5$; **, $p<0.05$.

2. Dclk1 isoforms were expressed differentially in gastric mucosa of DMP-777 treated mouse

The Dclk1 gene contains two N-terminal doublecortin(DC) domains, serine/proline-rich domain in between DC and kinase domain, and a C-terminal protein kinase domain (Fig. 2).[8,26] It has multiple splice variants resulting from alternative splicing, mainly full length isoform (LF), an isoform with only the doublecortin domain (363), and an isoform with only the kinase domain (SF). In case of mouse, 3 isoforms are produced; LF, SF, and 363.

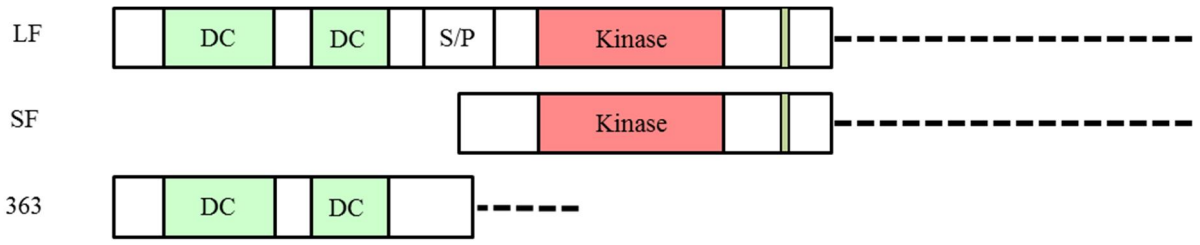

Figure 2. Alternative splice products of the Dclk1. LF contain two N-terminal doublecortin domains, serine/proline-rich domain, and C-terminal protein kinase domain. SF has only C-terminal kinase domain and 363 isoform has only DC domain.

Given the expression of Dclk1 increased with emergence of SPEM, and since Dclk1 has multiple isoforms, it was hypothesized that the expression of Dclk1 isoforms would alter in association with metaplasia and gastric carcinogenesis. To test this hypothesis, SPEM was induced by DMP-777 and the expression of Dclk1 isoforms was evaluated by western blotting and qPCR analysis. DMP-777 was administered orally as a gavage (350 mg/kg/day) once daily until sacrifice for 3 days, 7days, 14 days, 28 days and cessation after 14 days of treatment. After sacrifice, stomach was opened along the greater curvature and punctured into 12 regions (Fig. 3). Protein expression of Dclk1 isoforms was analyzed regionally in time-dependent manner (Fig. 4). The most noticeable finding was that the expression of LF was relatively stronger than SF in all regions. In limiting ridge regions (R1-R6), a junction between forestomach and glandular stomach where Dclk1 is highly expressed, both LF and SF expression increased by 3 days of DMP-777 treatment and gradually increased even after 7 days and up to 14 days of DMP-777 treatment. These expressions returned to normal level when DMP-777 was withdrawn and left to recovery for 14 days. In fundic regions (F7-F10) where SPEM develops, sharp increase of SF expression was detected by 3 days of DMP-777 treatment and its expression stayed elevated during the rest of the DMP-777 treatment period. As opposed to limiting ridge and fundic regions, reduced expression of LF and SF was rather observed in antrum (A11 and A12). These findings indicate that Dclk1 isoforms are expressed differentially during metaplasia and also expressed differentially according to the regions of the stomach.

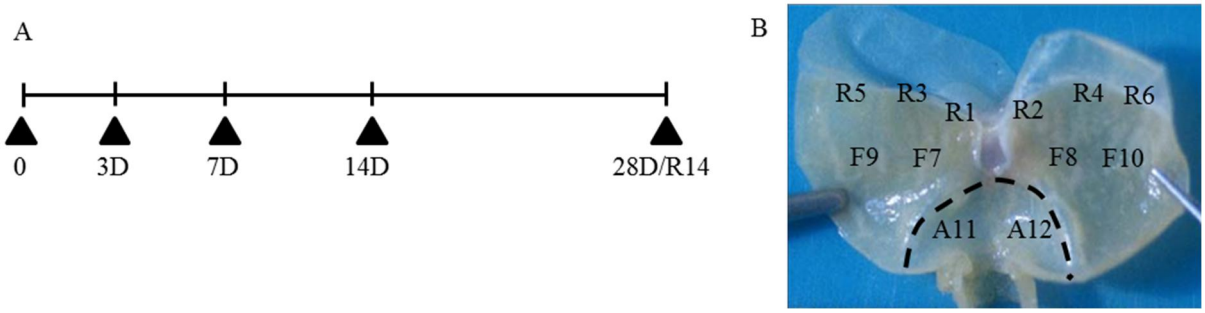

Figure 3. Schematic outline of DMP-777 treatment. (A) DMP-777 was administered orally as a gavage (350 mg/kg/day) once daily until sacrifice. (B) After sacrifice, stomach of DMP-777 treated mouse was opened along the greater curvature and punctured into 12 regions. R1-R6 is punctured along the limiting ridge. F7-F10 is within the fundus and A11, A12 are antrum.

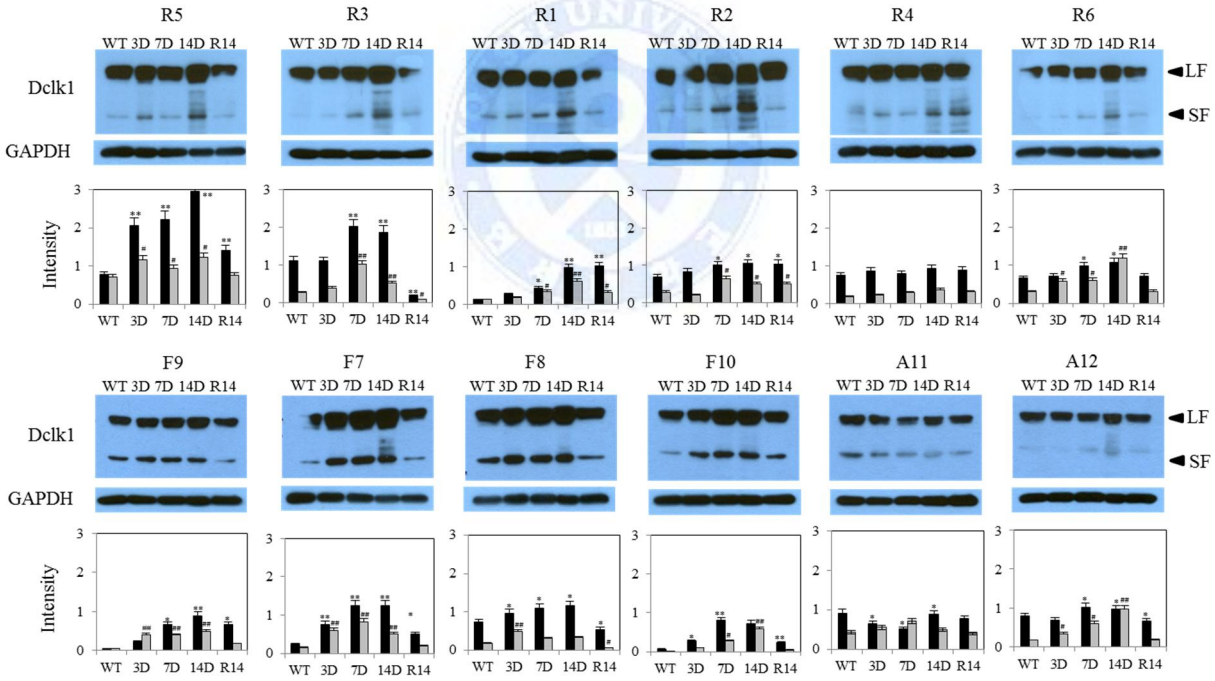

Figure 4. Protein expression of Dclk1 isoforms in DMP-777 treated mouse stomach. Tissue lysates of 12 regions in DMP-777 treated stomach were analyzed time-dependently by western blot. In limiting ridge regions (R1-R6), both LF and SF increased as DMP-777 was treated. In fundic regions (F7-F10), sharp increase of SF was observed by day 3 and continued for 7 days and 14 days of DMP-777

treatment. However, reduced expression of SF was rather observed in antrum (A11 and A12). Representative experiments were repeated 5 times. Intensity was measured by imageJ. Values are expressed as mean ± SEM. For LF, vs WT *, $P<0.05$; **, $P< 0.01$. For SF, vs WT #, $P<0.05$; ##, $P<0.01$.

3. The gene expression of Dclk1 isoforms showed dynamic spatial pattern

To investigate whether expressional variations of Dclk1 in translational level is derived from their transcritipnal regulation, the mRNA levels of Dclk1 isoforms were determined (Fig. 5). In contrast to translational level, RNA expression was not organized according to regions. Rather, the expression of all 3 isoforms varied in all 12 regions. For LF, expression could be classified into 3 groups, red, blue, and black bar. Group represented in Red bar showed similar pattern in which expression of LF decreased by day 3 and increase on day 7 of DMP-777 treatment. Groups in Blue bar showed increased LF expression by 3 days of DMP-777 treatment. Other regions shown in black bar did not have any distinct patterns. Surprisingly, when DMP-777 was treated continuously for 28 days, LF expression went down beyond normal level. These changes were recovered to normal level when DMP-777 was withdrawn after 14 days of treatment. SF expression was sorted into 2 groups. Regions that showed decreased SF expression by day 3 were assigned in green bar, and group that had no detectable changes were classified in gray bar. On the contrary to LF, increased level of SF was observed in all 12 regions when DMP-777 was continuously administered for 28days, and returned to normal level in all regions after recovery for 14 days. No significant changes were observed for 363 isoform whether DMP-777 was treated for 3 days, 7 days, 14 days, 28 days, and left to recovery for 14 days. In these studies, the transcriptional level of Dclk1 isoforms was not completely consistent with its translational level. Also, these data confirm the alteration of Dclk1 isoform expressions during metaplasia stage of gastric carcinogenesis.

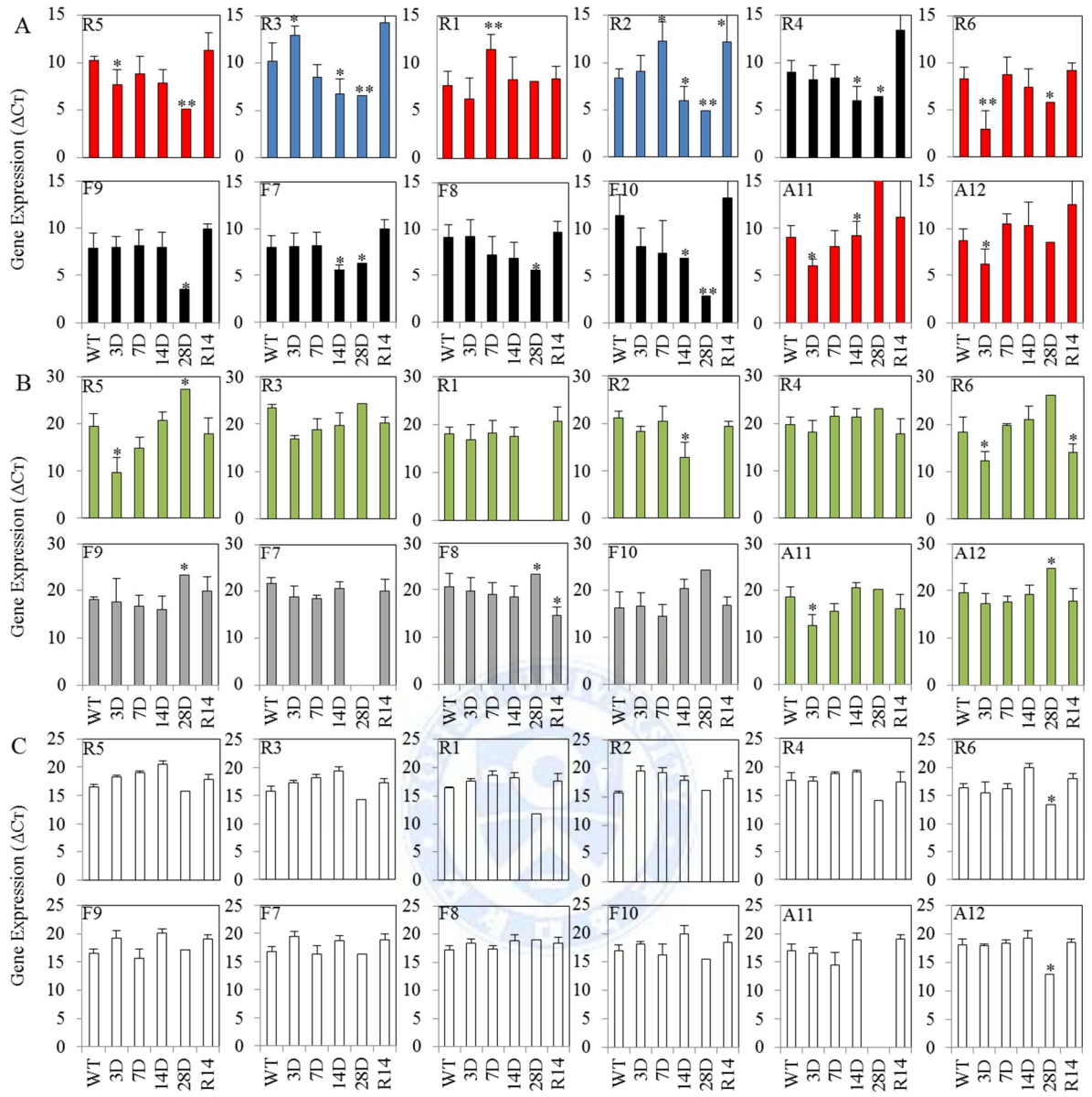

Figure 5. Gene expression of Dclk1 isoforms in DMP-777 treated mouse stomach. The levels of Dclk1 isoforms varied in all 12 regions. LF (A) was classified into three groups, red, blue, and black bar, and SF (B) was sorted into two groups, green and gray bar. No significance was observed for 363 (C) isoform except during 28 days of DMP-777 treatment in R6 and A12. Representative experiments were repeated 5 times. Values were expressed as mean ± SEM. vs WT. *, P<0.05; **, P< 0.01.

4. Each Dclk1 isoform identified different region within the cell

Through western blotting and qPCR analysis, it was supported that the expression of Dclk1 isoforms vary as metaplasia was induced. Gastric mucosa is composed of heterogeneous group of cells. Therefore, cellular localization of Dclk1 isoforms was characterized by immunofluorescence analysis (Fig. 6). Immunofluorescence was performed in gastric mucosa of wild-type and 14 days DMP-777 treated mouse with two different antibodies that recognizes different epitopes of doublecortin (DC) and kinase domains of Dclk1, respectively (Fig. 6A). As seen in Fig. 1, increased number of Dclk1-positive cells was observed in 14 days DMP-777 treated mouse gastric mucosa. Most of the cells that are positive for Dclk1 were shown in white, indicating LF expressing cells. This is in fact in parallel with western blot analysis because the intensity of LF expression was stronger than SF in all regions of the stomach. Another interesting finding was that the distribution of two isoforms differed even within the same cell (Fig. 6B). DC-positive signal was detected on the membrane while kinase signal was positive in cytoplasm.

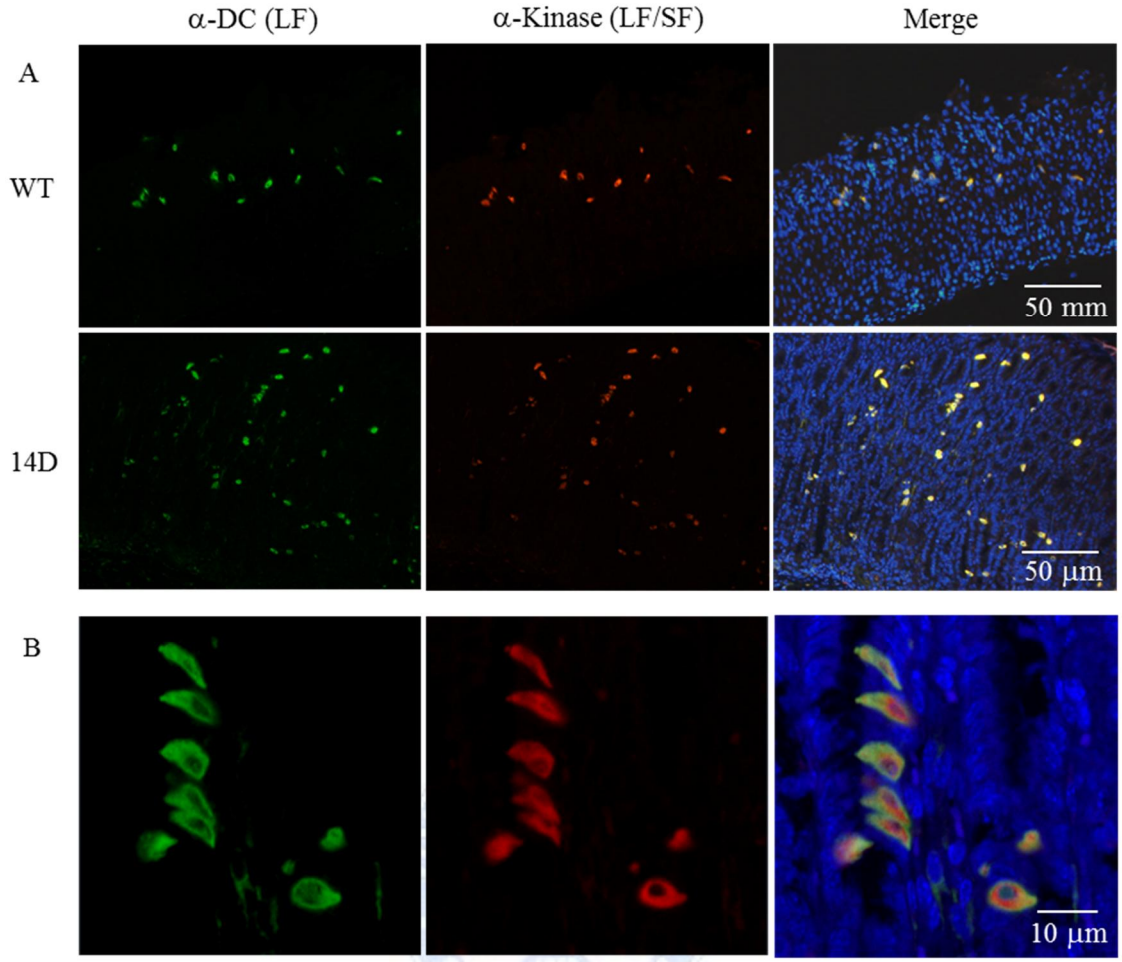

Figure 6. Immunofluorescence analysis of Dclk1-expressing cells in DMP-777 treated gastric mucosa. (A) Wild-type and 14 days DMP-777 treated mouse stomach sections were immunostained with two kinds of antibodies against Dclk1. α-DC (green) indicates LF and α-Kinase (Red) indicates both LF and SF. Increased number of Dclk1 expressing cells were observed as SPEM was induced by DMP-777. (B) DC signal was detected on the membrane while Kinase signal was detected in cytosol. Nuclei were stained with DAPI. White= LF, red only= SF, Green only= 363.

5. Dclk1-expressing cells expressed tuft cell marker

To examine whether Dclk1-positive cells associated with parietal cell loss express tuft cell marker, immunofluorescence analysis was performed using the two Dclk1 antibodies and tuft cell marker, acetylated-α-Tubulin (AcTub) (Fig. 7). In both wild-type and 14 days DMP-777 treated mouse gastric

mucosa, majority of the Dclk1-expressing cells, regardless of either LF or SF, co-expressed Dclk1 and AcTub in apical microvilli. These cells were confirmed to be tuft cells by their morphology and expression of AcTub. This analysis provides that Dclk1-expessing cells that increase in number in association with SPEM are highly correlated with tuft cell.

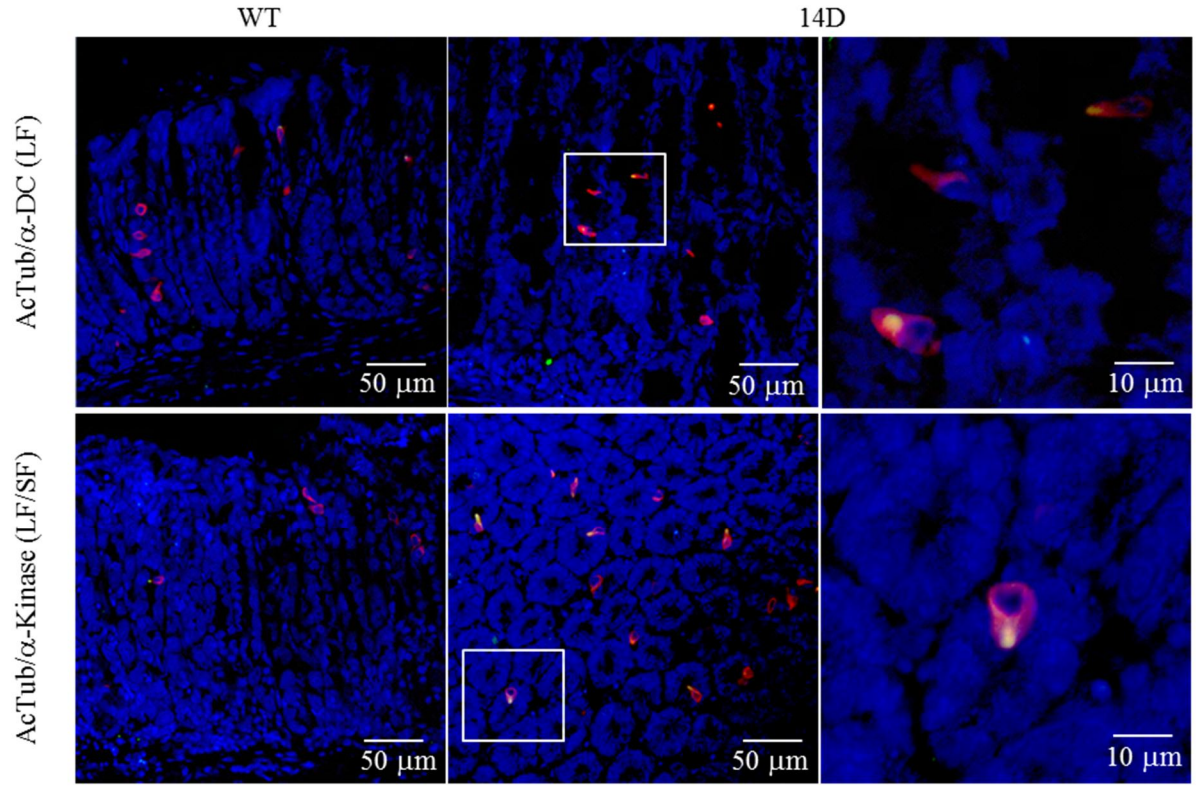

Figure 7. Immunofluorescence staining of Dclk (red) and acetylated-α-Tubulin (acTub, green) in metaplastic gastric mucosa. Co-localization of Dclk1 and acTub was observed at the apical region. These signals indicate microvilli of tuft cells. Nuclei were stained with DAPI.

6. *In situ* hybridization of Dclk1 isoforms in mouse SPEM model

Based on findings through protein and RNA analysis, it was noticed that the expression of Dclk1 isoforms differ in translational and transcriptional level. To confirm the localization of Dclk1 RNA expression, dual fluorescence *in situ* hybridization was performed (Fig. 8). Due to high homologies among the isoforms, it was hard to design probe for each isoform. Thus, DC probe that identifies

doublecortin domain which implies LF was designed to express red and kinase probe that identifies kinase domain indicating both LF and SF was labeled with green fluorescein. Therefore, when DC and kinase probe overlap, it would appear in white, indicating LF. If only red is expressed, it implies 363 isoform, and if only green is expressed, it would be SF. Using these DIG-labeled or/and fluorescein-labeled probe that accords with each domain of Dclk1, *in situ* hybridization was performed. In normal gastric mucosa, few cells in white which are LF expressing cells were observed mostly at the base of the gland. When DMP-777 was treated for 7 days, more of LF-expressing cells were scattered throughout the base of the gland. In addition, strong green positive SF-expressing cells started to appear in the isthmus region. By 14 days of DMP-777 treatment, these cells were much more abundant in the isthmus region. While no changes of LF and SF were observed in immunohistochemical analysis, in RNA level, it was supported that isoforms of Dclk1 are indeed involved in gastric carcinogenesis and that SF may play a role in SPEM progression

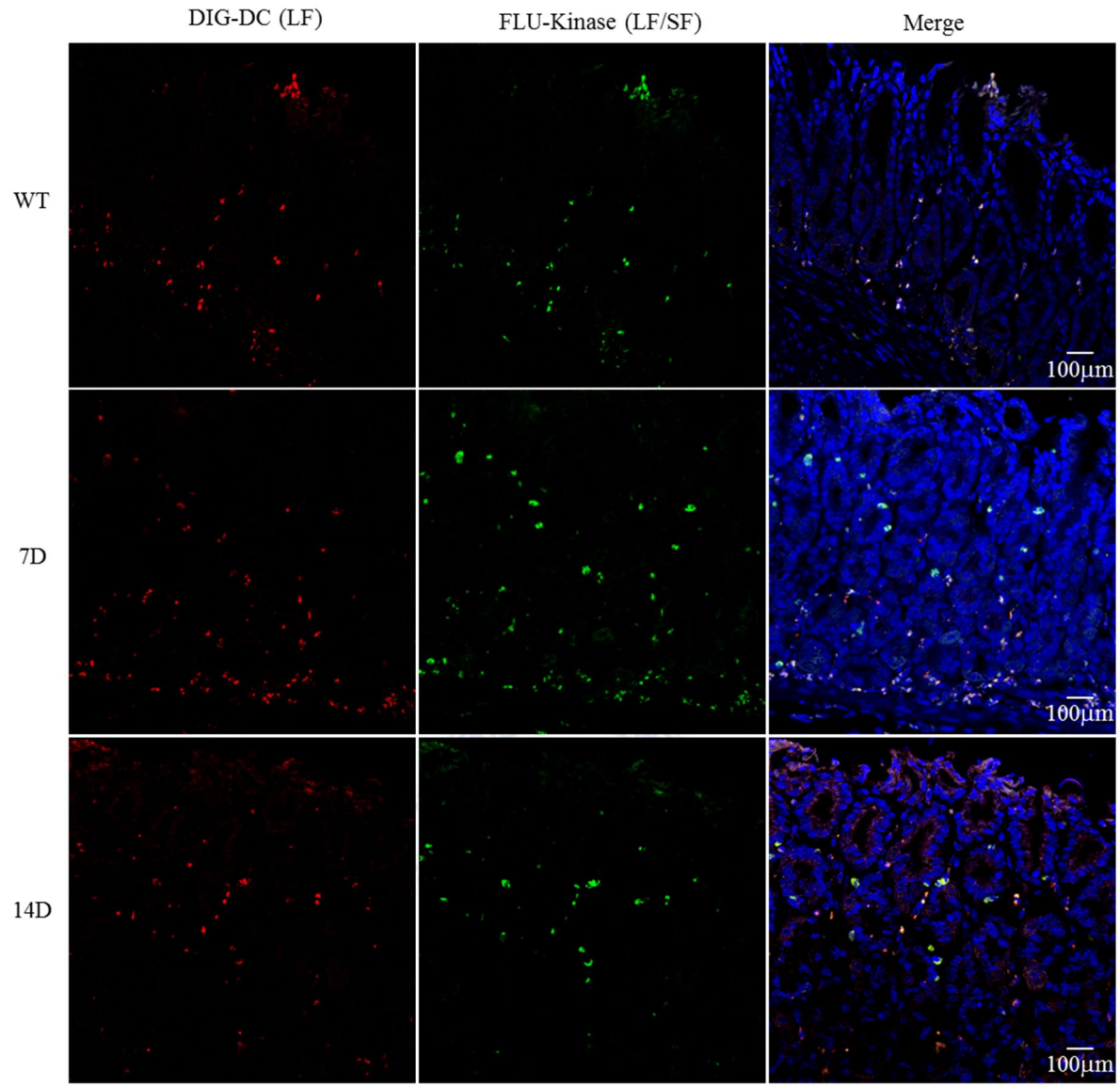

Figure 8. Dual fluorescence *in situ* hybridization of Dclk1 isoforms. Dual fluorescence *in situ* hybridization was performed using DIG-labeled probe (doublecortin domain) which was detected by rhodamin-labeled anti-DIG IgG or fluorescein-labeled probe (kinase domain) to characterize localization of Dclk1 isoforms within the gastric mucosa of DMP-777 treated mice. In wild-type, few cells co-positive for DC and Kinase domains were observed mostly at the base of the gland. By 7 days of DMP-777 treatment, stronger SF expressing cells started to appear at the isthmus neck region and increased in number by 14 days of DMP-777. Nuclei were stained with DAPI.

7. Expression of Dclk1 isoforms in tissues of gastric cancer patients

Next, to examine the changes of Dclk1 isoforms during human gastric carcinogenesis, endoscopic resection specimens of gastric cancer patients with areas of normal (N), intestinal metaplasia (IM), and cancer (C) were obtained and used to analyze the expression of Dclk1 isoforms by western blot and qPCR (Fig. 9). Although it was all different individual, similar expression pattern was observed. In all cases, LF and SF were markedly expressed in N and IM. However, in cancer, LF expression was dramatically lost and only SF was expressed (Fig. 9A). In parallel to mouse study, the gene expression did not correspond with protein expression. The expression of LF significantly increased in IM and C, whereas, SF significantly decreased in IM and C (Fig. 9B). These findings suggest important role of Dclk1 LF when metaplasia is further progressed to cancer.

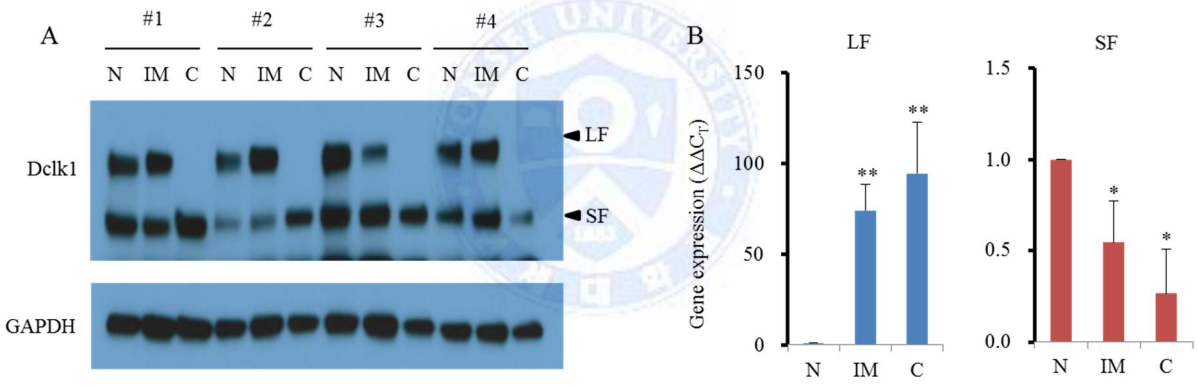

Figure 9. Expression of Dclk1 isoforms in endoscopic resection specimens of gastric cancer patients. (A) Immunoblot analysis of Dclk1 LF and SF expression was remarkable in N and IM, but LF was dramatically lost in C. (B) Relative expression of Dclk1 LF and SF in N, IM, and C assessed by qPCR. LF increased significantly in IM and C while SF decreased following IM and C. Values are expressed as mean ± SEM. vs N. *, $P<0.05$; **, $P< 0.01$.

8. Cellular localization of Dclk1 in human gastric tissues by *in situ* hybridization

The greatest discrepancy between mRNA and protein expression was seen for Dclk1 LF. As a mean to confirm this discrepancy, the RNA expression of Dclk1 LF in human gastric tissues was

performed by *in situ* hybridization using DIG-labeled probe (Fig. 10). It was found that LF expression was highly expressed in the isthmus region of normal tissue. Its expression was also detected in SPEM, IM and cancer. In IM, strong LF positive signal was observed in goblet cells. In cancer tissues, the signal was not positive in the goblet cells or other areas but only detected in cancerous region.

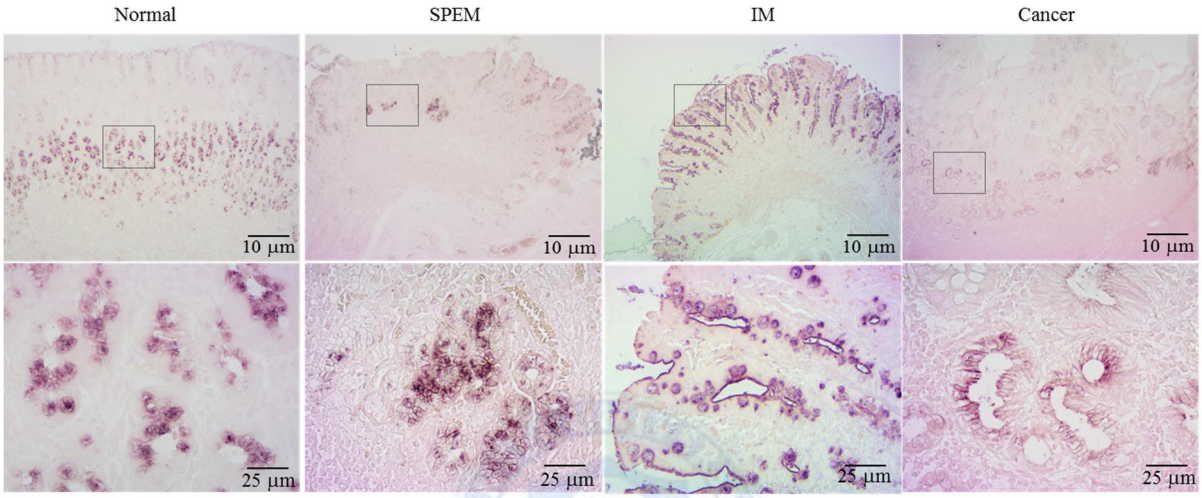

Figure 10. Cellular localization of Dclk1 LF in human gastric tissues. Cellular localization of Dclk1 LF in human gastric tissues was analyzed by *in situ* hybridization using DIG-labeled DC probe. Counterstained with nuclear fast red.

IV. DISCUSSION

Dclk1 is known as a putative intestinal stem cell marker[10,11] as well as a tuft cell marker.[12] Recently, its function as a cancer stem cell has been identified, raising a great interest in relevance of Dclk1 and cancer.[17] Dclk1 has multiple isoforms generated from alternative splicing, including LF, SF, and 363 isoform.[8] Several previous studies have investigated the expression of Dclk1 isoforms in developing nervous system.[8,27,28] It has been demonstrated that both LF and SF are mainly expressed in human fetal brain and in low levels of adult tissues.[27,28] Indeed, the preliminary western blotting data confirmed the high expression of both LF and SF in brain as well as in various organs of normal mouse, although there were minor intensity differences.

Dclk1 has been shown to be overexpressed in various cancers including esophageal, gastric, colorectal, pancreatic, breast and prostate compared to paired normal tissues.[16,23,29,30] Nevertheless, none of these studies have addressed the question on the isoforms of Dclk1 in relation with cancer. Interestingly, in the preliminary data, when gastric cancer cell lines were examined for Dclk1, only SF was detected compared to non-cancer cell lines which expressed both LF and SF. This finding was repeated in other cancer cell lines including colon as well as pancreatic cancer cell lines, suggesting that Dclk1 isoforms are somewhat involved in the course of gastric carcinogenesis. Thus, the present study tried to identify the expression of Dclk1 isoforms during gastric carcinogenesis.

Gastric cancer develops through a number of stages including inflammation, gastritis, and metaplasia.[1] Metaplasia is precancerous stage before proceeding to invasive cancer. Here, mouse SPEM model induced by DMP-777, which causes oxyntic atrophy, was used to investigate the expression of Dclk1 isoforms.[1,5] As recently reported by Choi et al.,[16] treatment of DMP-777 increased the number of Dclk1-expressing cells only in the fundus.

The normal gastric mucosa is composed of various types of cells that have a unique geographical distribution.[31-36] Progenitor cells are located in the isthmus-neck region and give rise to acid-secreting parietal cells, mucous neck cells, and zymogen-secreting chief cells.[3,4] The gene expression might vary between regions due to its cellular composition. To resolve this unique distribution of different

cell types, the stomach was divided into 12 different regions and the regional expression of Dclk1 isoforms was measured. It was found that the expression of LF was more abundant than SF. In translational level, both isoforms increased as metaplasia was induced in limiting ridge and fundus, however, no changes were detected in antrum. In contrast, mRNA expression of each isoforms varied in all regions. This difference in protein and RNA level was further confirmed by immunohistochemical analysis. One of the interesting finding was the different spatial location of LF and SF in the same cell. In addition, through *in situ* hybridization, it was clearly seen that RNA expression of Dclk1 isoform are shifted from LF to SF during SPEM progression which represents the first definite expression difference of Dclk1 isoforms in association with oxyntic atrophy.

Dclk1-expressing cells exhibit unique feature that resembles the tuft cells.[12] These tuft cells are reported to be increased with inflammation, hyperplasia, and metaplasia.[14,37] Given the evidence that Dclk1-expressing tuft cells expand in association with gastric tumor,[16,22] similar observation of colocalization of tuft cell marker, acTub with Dclk1 was detected in apical region. Moreover, these cells were not confined to either LF or SF.

The most interesting outcome of this study was observed in the human gastric cancer patients' sample. Biopsies of gastric cancer patients were obtained and examined for the expression of Dclk1. Surprisingly, while LF and SF existed notably in N and IM, LF was lost and only SF was expressed in cancer. These findings are somewhat consistent with results of previous study showing an increased expression of Dclk1 during early stage metaplasia of ADM and PanIN, and loss of these expressions in invasive pancreatic cancer.[23] Beyond just expression in cancer, current study confirms the alteration of Dclk1 isoforms in gastric carcinogenesis. Moreover, this novel finding suggests Dclk1 LF as a tumor suppressor role in the course of gastric carcinogenesis. More importantly, in agreement to mouse study, the protein and RNA expression did not show any similar trend. Such inconsistency is thought to result from post-transcriptional modification especially by microRNA. Usually, microRNAs bind to 3'-UTR and repress the expression of target gene. However, microRNA can also bind to 5'-UTR of target gene and not disturb the expression of target gene. Further investigation regarding microRNA will provide greater insight into regulation of Dclk1 isoforms in progression of

SPEM as well as in gastric cancer.

In summary, most perspectives of Dclk1 are just focused on its expression in cancer. The present investigations have not only centered on its expression but also the isoform changes in association with gastric carcinogenesis. The finding of strong SF during SPEM progression as well as loss of LF in cancer supports the concept of an alteration of Dclk1 isoform expressions in gastric cancer progression. Targeting these cell populations may have therapeutic potential in the prognosis, treatment and/or chemoprevention of gastric cancer.

V. CONCLUSION

The study showed that Dclk1-expressing cells increased in number in assoication with parietal cell loss. Given the multiple splice variants of Dclk1, the expression of Dclk1 isoforms during gastric carcinogenesis have been examined. It was found that the expression of Dclk1 isoforms are altered in gastric carcinogenesis. SPEM was induced by DMP-777 and the expression of Dclk1 isoforms were examined in translational and transciprtional elvel. While the expression intensity of both LF and SF increased in metaplastic gastric muscosa, the expression of mRNA was dynamic defined to regional location. One intersting finding was that each isofrom was labeled differently even within the same cell. Additionally, *in situ* hybridization analysis confirmed the altered expression of Dclk1 isoforms in metaplsia by oxyntic atrophy. These findings imply possibilities that Dclk1 isoforms play an important role in gastric carcinogenesis. Furthermore, it was seen that from endoscopic resection specimens of gastric cancer patients, LF was lost and only SF was expressed in cancer. This finding suggest Dclk1 LF as a hall marker in determining the progression of gastric cacner. Altogether, these data provide new cellular and molecular aspects of Dclk1 isoforms in association with different stages of gastric cancer.

REFERENCES

1. Goldenring JR, Nam KT. Oxyntic atrophy, metaplasia, and gastric cancer. Prog Mol Biol Transl Sci 2010;96:117-31.
2. Nam KT, Lee HJ, Sousa JF, Weis VG, O'Neal RL, Finke PE, et al. Mature chief cells are cryptic progenitors for metaplasia in the stomach. Gastroenterology 2010;139:2028-37 e9.
3. Nam KT, O'Neal RL, Coffey RJ, Finke PE, Barker N, Goldenring JR. Spasmolytic polypeptide-expressing metaplasia (SPEM) in the gastric oxyntic mucosa does not arise from Lgr5-expressing cells. Gut 2012;61:1678-85.
4. Nozaki K, Ogawa M, Williams JA, Lafleur BJ, Ng V, Drapkin RI, et al. A molecular signature of gastric metaplasia arising in response to acute parietal cell loss. Gastroenterology 2008;134:511-22.
5. Goldenring JR, Ray GS, Coffey RJ, Meunier PC, Haley PJ, Barnes TB, et al. Reversible drug-induced oxyntic atrophy in rats. Gastroenterology 2000;118:1080-93.
6. Li Q, Karam SM, Gordon JI. Diphtheria toxin-mediated ablation of parietal cells in the stomach of transgenic mice. J Biol Chem 1996;271:3671-6.
7. Weis VG, Sousa JF, LaFleur BJ, Nam KT, Weis JA, Finke PE, et al. Heterogeneity in mouse spasmolytic polypeptide-expressing metaplasia lineages identifies markers of metaplastic progression. Gut 2013;62:1270-9.
8. Omori Y, Suzuki M, Ozaki K, Harada Y, Nakamura Y, Takahashi E, et al. Expression and chromosomal localization of KIAA0369, a putative kinase structurally related to Doublecortin. J Hum Genet 1998;43:169-77.
9. Lin PT, Gleeson JG, Corbo JC, Flanagan L, Walsh CA. DCAMKL1 encodes a protein kinase with homology to doublecortin that regulates microtubule polymerization. J Neurosci 2000;20:9152-61.
10. Giannakis M, Stappenbeck TS, Mills JC, Leip DG, Lovett M, Clifton SW, et al. Molecular properties of adult mouse gastric and intestinal epithelial progenitors in their niches. J Biol

Chem 2006;281:11292-300.

11. May R, Riehl TE, Hunt C, Sureban SM, Anant S, Houchen CW. Identification of a novel putative gastrointestinal stem cell and adenoma stem cell marker, doublecortin and CaM kinase-like-1, following radiation injury and in adenomatous polyposis coli/multiple intestinal neoplasia mice. Stem Cells 2008;26:630-7.
12. Gerbe F, Brulin B, Makrini L, Legraverend C, Jay P. DCAMKL-1 expression identifies Tuft cells rather than stem cells in the adult mouse intestinal epithelium. Gastroenterology 2009;137:2179-80; author reply 80-1.
13. Sato A. Tuft cells. Anat Sci Int 2007;82:187-99.
14. Saqui-Salces M, Keeley TM, Grosse AS, Qiao XT, El-Zaatari M, Gumucio DL, et al. Gastric tuft cells express DCLK1 and are expanded in hyperplasia. Histochem Cell Biol 2011;136:191-204.
15. Gagliardi G, Bellows C. DCLK1 expression in gastrointestinal stem cells and neoplasia. journal of Cancer Therapeutics and Research 2012:12.
16. Choi E, Petersen CP, Lapierre LA, Williams JA, Weis VG, Goldenring JR, et al. Dynamic Expansion of Gastric Mucosal Doublecortin-Like Kinase 1-Expressing Cells in Response to Parietal Cell Loss Is Regulated by Gastrin. Am J Pathol 2015.
17. Nakanishi Y, Seno H, Fukuoka A, Ueo T, Yamaga Y, Maruno T, et al. Dclk1 distinguishes between tumor and normal stem cells in the intestine. Nat Genet 2013;45:98-103.
18. Burgess HA, Reiner O. Alternative splice variants of doublecortin-like kinase are differentially expressed and have different kinase activities. J Biol Chem 2002;277:17696-705.
19. Jin G, Ramanathan V, Quante M, Baik GH, Yang X, Wang SS, et al. Inactivating cholecystokinin-2 receptor inhibits progastrin-dependent colonic crypt fission, proliferation, and colorectal cancer in mice. J Clin Invest 2009;119:2691-701.
20. Kikuchi M, Nagata H, Watanabe N, Watanabe H, Tatemichi M, Hibi T. Altered expression of a putative progenitor cell marker DCAMKL1 in the rat gastric mucosa in regeneration, metaplasia and dysplasia. BMC Gastroenterol 2010;10:65.

21. Zhang Y, Huang X. Investigation of doublecortin and calcium/calmodulin-dependent protein kinase-like-1-expressing cells in the mouse stomach. J Gastroenterol Hepatol 2010;25:576-82.

22. Nam KT, O'Neal R, Lee YS, Lee YC, Coffey RJ, Goldenring JR. Gastric tumor development in Smad3-deficient mice initiates from forestomach/glandular transition zone along the lesser curvature. Lab Invest 2012;92:883-95.

23. Bailey JM, Alsina J, Rasheed ZA, McAllister FM, Fu YY, Plentz R, et al. DCLK1 marks a morphologically distinct subpopulation of cells with stem cell properties in preinvasive pancreatic cancer. Gastroenterology 2014;146:245-56.

24. Nomura S, Yamaguchi H, Ogawa M, Wang TC, Lee JR, Goldenring JR. Alterations in gastric mucosal lineages induced by acute oxyntic atrophy in wild-type and gastrin-deficient mice. Am J Physiol Gastrointest Liver Physiol 2005;288:G362-75.

25. Yu SS, Takenaka O. Molecular cloning, structure, and testis-specific expression of MFSJ1, a member of the DNAJ protein family, in the Japanese monkey (Macaca fuscata). Biochem Biophys Res Commun 2003;301:443-9.

26. Ishikawa K, Nagase T, Nakajima D, Seki N, Ohira M, Miyajima N, et al. Prediction of the coding sequences of unidentified human genes. VIII. 78 new cDNA clones from brain which code for large proteins in vitro. DNA Res 1997;4:307-13.

27. Mizuguchi M, Qin J, Yamada M, Ikeda K, Takashima S. High expression of doublecortin and KIAA0369 protein in fetal brain suggests their specific role in neuronal migration. Am J Pathol 1999;155:1713-21.

28. Matsumoto N, Pilz DT, Ledbetter DH. Genomic structure, chromosomal mapping, and expression pattern of human DCAMKL1 (KIAA0369), a homologue of DCX (XLIS). Genomics 1999;56:179-83.

29. Sureban SM, May R, Mondalek FG, Qu D, Ponnurangam S, Pantazis P, et al. Nanoparticle-based delivery of siDCAMKL-1 increases microRNA-144 and inhibits colorectal cancer tumor growth via a Notch-1 dependent mechanism. J Nanobiotechnology 2011;9:40.

30. Vega KJ, May R, Sureban SM, Lightfoot SA, Qu DF, Reed A, et al. Identification of the

putative intestinal stem cell marker doublecortin and CaM kinase-like-1 in Barrett's esophagus and esophageal adenocarcinoma. Journal of Gastroenterology and Hepatology 2012;27:773-80.

31. Karam SM, Leblond CP. Dynamics of epithelial cells in the corpus of the mouse stomach. I. Identification of proliferative cell types and pinpointing of the stem cell. Anat Rec 1993;236:259-79.

32. Karam SM, Leblond CP. Dynamics of epithelial cells in the corpus of the mouse stomach. II. Outward migration of pit cells. Anat Rec 1993;236:280-96.

33. Karam SM, Leblond CP. Dynamics of epithelial cells in the corpus of the mouse stomach. III. Inward migration of neck cells followed by progressive transformation into zymogenic cells. Anat Rec 1993;236:297-313.

34. Karam SM. Dynamics of epithelial cells in the corpus of the mouse stomach. IV. Bidirectional migration of parietal cells ending in their gradual degeneration and loss. Anat Rec 1993;236:314-32.

35. Karam SM, Leblond CP. Dynamics of epithelial cells in the corpus of the mouse stomach. V. Behavior of entero-endocrine and caveolated cells: general conclusions on cell kinetics in the oxyntic epithelium. Anat Rec 1993;236:333-40.

36. Choi E, Roland JT, Barlow BJ, O'Neal R, Rich AE, Nam KT, et al. Cell lineage distribution atlas of the human stomach reveals heterogeneous gland populations in the gastric antrum. Gut 2014;63:1711-20.

37. Morroni M, Cangiotti AM, Cinti S. Brush cells in the human duodenojejunal junction: an ultrastructural study. J Anat 2007;211:125-31.

< ABSTRACT (IN KOREAN)>

위암 발생과정에서 Dclk1 isoform 발현의 역동적 변형에 대한 연구

<지도교수 남 기 택>
연세대학교 대학원 의과학과
황 혜 경

Dclk1은 장에서 quiescent stem cell marker로 알려져 있을 뿐만 아니라, 위의 tuft cell의 마커로 알려져 있다. Dclk1은 long form(LF, full length), short form(SF, kinase domain only), 363 isoform (doublecortin domain only)의 3개의 전사체를 가진다. 선행연구에서 cancer와 Dclk1 발현간의 상관관계가 알려져 왔으나, metaplasia와 위암에서 Dclk1 isoform 발현에 대해서는 보고된 바가 없다. 이에 Dclk1의 isoform 발현이 위암 발생 과정에 관여할 것이라 가정하에 DMP-777을 투여한 마우스 SPEM 모델을 이용하여 Dclk1의 isoform 발현을 단백질과 RNA level에서 분석하였다. LF과 SF의 단백질 발현은 위의 전체 부위에서 증가하는 양상을 보였으나, 이에 반하여 RNA의 발현은 부위별로 다양한 발현양상의 변화를 보였다. 또 Dclk1 발현세포를 immunohistochemistry와 *in situ* hybridization 방법으로 관찰한 결과, Dclk1 isoform마다 세포 내에 발현하는 위치가 다를 뿐만 아니라 위암 발병 과정 중 Dclk1 isoform에 변화가 보이는 것을 확인하였다. 특히 metaplasia가 일어나면서 정상 gastric mucosa에서는 보이지 않던 SF을 강하게 발현하는 cell들이 isthmus region에서 확인되었다. 또 위암 환자의 조직에서도 Dclk1 isoform 발현의 차이를 확인해본 결과, 정상 조직과 intestinal metaplasia (IM) 조직에서는 LF과 SF 모두 강한 발현을 보인 반면에 비해 cancer 조직에서는 LF의 발현이 소실되고 SF만 발현하는 특징을 확인하였다. 이상의 결과는 Dclk1 isoform중 LF의 발현이 위암의 발생 과정에서 매우 중요할 것으로 사료된다.

핵심 되는 말: Dclk1, gastric carcinogenesis, SPEM, DMP-777

PUBLICATION LIST

1. Lee K, Hwang H, Nam KT. Immune response and the tumor microenvironment: how they communicate to regulate gastric cancer. Gut Liver 2014;8:131-9.
2. Yu S, Hwang HE, Yun N, Goldenring JR, Nam KT. The mRNA and Protein Levels of Tubulin and beta-Actin Are Greatly Reduced in the Proximal Duodenum of Mice Relative to the Rest of the Small Intestines. Dig Dis Sci 2015.