Expression of EGFR negative regulator, Lrig1 in gastric carcinogenesis

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Expression of EGFR negative regulator, Lrig1 in gastric carcinogenesis

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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

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June 2016

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June 2016

ACKNOWLEDGEMENTS

I would like to express my deep gratitude to Professor Ki Taek Nam for providing me the golden opportunity to do this thesis. He gave me a lot of support, encouragement and passion. This accomplishment would not have been possible without him. I am really thankful to him.

I would also like to acknowledge with much appreciation to Professor Kun Hong Kim, Professor Jun-Young Seo. I am gratefully indebted to their very valuable time, comments and advices on this thesis.

I sincerely thank Dr. Yu for her guidance, and immense knowledge in this study. She helped me in all time of this thesis.

I also wish to express my gratitude to my colleagues who rendered their help during the period of the study.

Finally, I must express my very profound gratitude to my family and to my friends for providing me with support,continuous love and encouragementall time.

Mijeong Yang

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<ABSTRACT>

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Leucine-rich repeats and immunoglobulin-like domains 1 (LRIG1) is a transmembrane protein that antagonises EGFR signaling by downregulating receptor levels. Recent studies have proposed that Lrig1 marks predominately non-cycling, long-lived stem cells located at the crypt +4 quiescent intestinal stem cells by lineage tracing. Multiple groups have reported that Lrig1 expression is downregulated in several cancers, including bladder, lung, renal, breast, and colorectal cancer. These results suggested that Lrig1, known as a tumor suppressor, plays an important role in maintaining intestinal epithelial homeostasis. However, the function of Lrig1 in gastric mucosa is not well understood yet. Here, I investigated the alternative Lrig1 expression using DMP-777 treated mouse SPEM model in gastric carcinogenesis by *in situ* hybridization. I also observed that the altered expression of Lrig1 during human gastric cancer development by *in situ* hybridization and immunohistochemistry. The results showed the Lrig1 expression was increased in SPEM lesion while it was lost in IM and gastric cancer lesion,

during gastric carcinogenesis. However, on the contrary to Lrig1, EGFR was upregulated throughout gastric carcinogenesis. Furthermore, I examined the effects of Lrig1 knockdown on proliferation of gastric cancer cell lines. The knockdown of Lrig1 promoted cell growth rate in gastric cancer cell lines. From our findings indicate Lrig1 as a negative regulator of EGFR, may act a critical point in metaplastic progression

Key words: Lrig1, gastric carcinogenesis, SPEM, IM

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I. INTRODUCTION

Gastric cancer is one of the most common cancer after lung, breast, colorectal and prostate in the world.[1] Gastric carcinogenesis is mediated through conformational change of gastric mucosa.[2] Chronic infection of *Helicobacter pylori*, which is designed as class Ⅰ carcinogen, is a major primary cause of gastric cancer in human.[2,3] Chronic *H.pylori* infection causes prominent inflammation and loss of parietal cell (or oxyntic atrophy).[4] Parietal cell loss (or oxyntic atrophy) appears as initiating event in gastric pre-neoplasia in humans because parietal cells play an important role in the differentiation of gastric mucosa.[4,5] In humans, oxyntic atrophy with the presence of inflammation can progress to two types of metaplasia: intestinal metaplasia (IM) and spasmolytic polypeptide-expressing metaplasia (SPEM).[5] Intestinal metaplasia is characterized by the presence of intestinal goblet cells in the stomach, which is not normally found in the normal stomach.[2,4,5] Intestinal metaplasia expresses intestinal markers Muc2, Trefoil factor 3 (TFF3), other intestinal brush border markers including sucrose isomaltase, vilin, Muc13 and fatty acid binding protein.[4,6] Another possible pre-neoplastic

metaplasia,spasmolytic polypeptide-expressing metaplasia (SPEM), displays similar morphological characteristics with deep antral glands or Brunner's glands but gastrin cells are not observed in SPEM gland. SPEM is characterized by appropriate markers including Muc6, Trefoil Factor 2 (TFF2).[2,4-7]

Recent investigations suggest that several mouse SPEM models can be experimentally induced by infection with *Helicobacter felis,* or treatment with DMP-777, or L-635. Chronic infection with *Helicobacter felis* for about 6 months results in loss of parietal cells and causes SPEM with an inflammation response.[8] Treatments with 3 daily dose of L-635, structurally related β-lactam compound, causes prominent parietal cell loss and induces SPEM in inflammation. [9] DMP-777 is a cell permeant neutrophil elastase inhibitor.[4] After 14 days of DMP-777 administration, mice develop oxyntic atrophy. Acute destruction of parietal cells leads to emergence of SPEM without inciting an inflammatory response. Moreover, these legions can recover back to normal state when DMP-777 is suspended after treatment.[9]

Leucine-rich repeats and immunoglobulin-like domains 1 (LRIG1),located at chromosome 3p14.3, is a transmembrane protein that has extracellular domain containing 15 leucine-rich repeats (LRRs) and 3 immunoglobulin (Ig)-like domains. It is capable of interacting with all four members of the ERBB receptor family and regulating receptor levels by enhancing receptor ubiquitylation and lysosomal degradation, independent of EGFR ligands.[10-12] Lrig1 was identified as human epidermal stem cell markers in a quiescent non proliferative state.[13] Genetic ablation of Lrig1 caused increased proliferation associated with stem cell expansion in vitro and epidermal hyperproliferation in vivo.[13,14] However, recent studies have proposed that Lrig1 marks predominately non-cycling, long-lived stem cells located at the crypt +4 quiescent intestinal stem cells by lineage tracing.[15] This study also

demonstrates that loss of *Apc* in Lrig1-positivecells leads to intestinal adenomas and results in heightened ErbB1-3 expression and duodenal adenomas.[15] In 2002, genetic ablation of Lrig1 in mouse model was first reported. Lrig1 null mice resulted in psoriasiform epidermal hyperplasia on tails, face and ears but tumor remained free.[16] Several groups observed that enlarged intestinal size and expansion of crypt with elevated levels of ErbB1-3 throughout the small intestine.[15,17] Levels of pEGFR, an activation form of EGFR, are low at the crypt bottom and peak around +8 position. Of note, this expression pattern is the reverse of Lrig1.[17] In human cancers, multiple groups have reported that the Lrig1 expression is down regulated in several cancers, including bladder, lung, renal, breast, and colorectal cancer.[18-22] These results suggested a potential role of Lrig1 as a tumor suppressor in maintaining intestinal epithelial homeostasis.

Several groups have reported the roles of Lrig1 in epithelial tissues such as the epidermis, small intestine and colon. However, little remains known about function of Lrig1 in gastric mucosa. In this study, I attempted to investigate the alternative Lrig1 expression using DMP-777 treated mouse SPEM model in gastric carcinogenesis and the altered expression of Lrig1 was analyzed in human gastric cancer patient. Furthermore, I examined the effects of Lrig1 knockdown on proliferation of gastric cancer cell lines. In our study, Lrig1 was observed in region where normal stem cells are known to reside in normal stomach. Lrig1 co-localized with proliferating cell marker and was strongly expressed in SPEM mouse model. During human gastric carcinogenesis, Lrig1 expression was enhanced in SPEM lesion while it was lost in IM and gastric cancer lesion. However, on the contrary to Lrig1, EGFR was upregulated throughout gastric carcinogenesis. Accordingly, our results indicate Lrig1 as a negative regulator of EGFR in gastric carcinogenesis.

II. MATERIALS AND METHODS

1. Cell Culture

MKN1, MKN28, SNU1, SNU638 were incubated in RPMI1640 medium with 10% fetal bovine serum and 1% penicillin/streptomycin. AGS were incubated in Ham′s F12K medium with 10% fetal bovine serum and 1% penicillin/streptomycin. Five gastric cancer cell lines were maintained at 37℃ in a humidified atmosphere of 5% CO_2.

2. Western Blotting

Harvested cells were lysed in lysis buffer (0.02M Tris(pH 7.4), 0.15M Nacl, 10% Glycerol) supplemented with 100XHalt™ Phosphatase Inhibitor Cocktail (Thermo scientific, Rockford, IL, USA). Lysates were collected by centrifugation at 14,000rpmfor 20min at 4℃. After quantifying the concentration of proteins with BCA protein assay (Pierce Biotechnology, Rockford, IL, USA), lysates were mixed with 1x sample buffer and boiled at 95°C for 10 minutes. Thirty-microgram of total proteins was separated on 7% SDS-PAGE gel and transferred to a PVDF membrane (Millipore, Billerica, MA, USA) at 4℃. After blocking with 5% skim milk, membranes were incubated with primary antibodies at 4℃ overnight followed by incubation with HRP conjugated secondary antibody at room temperature for 1 hr. The proteins were detected using ECL reagents (Bionote, Hawseong, Korea) and intensities of detected signals were quantified with Image J (developed by Wayne Rasband). The primary antibodies used were anti-Lrig1 (1:2000

dilution; Biorbyt, Cambridge, UK), anti-EGFR (1:100000 dilution; abcam, Cambridge, UK), and anti-Gapdh (1:20000 dilution; abcam, Cambridge, UK) and anti-rabbit HRP-conjugated antibody (1:4000 dilution; Life Technology, Carlsbad, CA, USA)

3. RNA extraction and Quantitative PCR (qPCR)

Total RNA was extracted from gastric cancer cells with TRIzol (Life Technology, Carlsbad, CA, USA). Using 1mg of DNase treated total RNA, cDNAs were synthesized using the ImProm-II™ reverse transcription system (Promega, Madison, WI, USA).Synthesized cDNAs were analyzed by POWER SYBR Green Master Mix (Applied Biosystems, Waltham, MA, USA), on 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
Lrig1_Fw	GTCACGGTCGCTGCTAACTC
Lrig1_Rv	TGGAAGGTGAGGCCCTCTAT
EGFR-Fw	ATGCCCGCATTAGCTCTTAG
EGFR-Rv	GCAACTTCCCAAAATGTGCC
Actin-Fw	AGAGCTACGAGCTGCCTGAC
Actin-Rv	AGCACTGTGTTGGCGTACAG
ISH-Lrig1-Fw	GGGTCGCTCTACCCCAGTA
ISH-Lrig1-Rv	TTCCATCCTTCCCACCCCG

4. Immunohistochemistry (IHC)

Sections from paraffin-embedded tissues were deparaffinized by histoclear and rehydrated through 100%, 95% and 70% ethanol. Using a pressure cooker, antigen retrieval was performed in the target retrieval solution (Dako,

Glostrup, Denmark)for 15 min. Endogenous peroxidase activity was blocked with 3% H_2O_2 for 30 minutes followed by protein blocking in protein block serum free ready to use solution (Dako, Glostrup, Denmark) for 1hr at room temperature. Primary antibody was diluted with antibody diluent solution (Dako, Glostrup, Denmark) and incubated in a humidified chamber at 4°Covernight.Slides were incubated with secondary rabbit IgG (Dako, Glostrup, Denmark) for 15 minutes at room temperature, and detected with Dako Envision+ System-HRP DAB (Dako, Glostrup, Denmark). Counterstained with Meyer′s Hematoxylin (Sigma-Aldrich, St. Louis, USA)for 5 min and dehydrated through 70%, 95% and 100% ethanol. The slides were mounted with mounting solution (Electron Microscopy Sciences, Hatfield, PA, USA). For immunofluorescence, Alexa 488 conjugated anti-rabbit secondary antibody (Life Technology, Carlsbad, CA, USA) was used. For dual staining of rabbit anti-Ki67 antibodies, TSA (Tyramide Signal Amplification)-Plus Fluoresce in 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). For dual staining after *in situ* hybridization, the slides were washed several times with 1X PBS, VECTASTAIN ABC reagent (Vector Laboratories, INC, Burlingame, CA, USA) was supplied according to the manufacturer's instructions. Antibodies against TFF2 (1:100 dilution; abcam, Cambridge, UK) and anti-mouse HRP-conjugated Ig M antibody (1:200 dilution; ZYMED, San Francisco, CA, USA) were used.

***5. In situ hybridization*(ISH)**

Four-micrometer sections from paraformaldehyde-fixed and paraffin-embedded tissue samples were deparaffinized and rehydrated, and then the tissue sections were incubated with 0.5% acetic anhydride solution to remove

nonspecific binding. Hybridization was performed with DIG-labeled sense and antisense cRNA probes in 2× SSC containing 50% formamide at 42 °C overnight. Next day, the slides were washed in SSC solution at 50℃and incubated with an anti-DIG Fab antibody (Roche Diagnostics, Indianapolis, IN, USA) conjugated to alkaline phosphatase or anti-DIG Rhodamine antibody (Roche Diagnostics, Indianapolis, IN, USA) at 4 °C overnight and detected with the addition of Nitro Blue Tetrazo lium/5-bromo-4-chloro-3-indolyl phosphate(Roche Diagnostics, Indianapolis, IN, USA). Nuclei were counterstained in Nuclear Fast Red (Vector Laboratories, Inc., Burlingame, CA, USA). Nucleotide 3045-3780 of Lrig1 sequence was amplified using PCR to synthesis cRNA probe. Primers used for PCR are provided in Table 1. The PCR products were cloned into the pGEM-TE vector and digested with restriction enzyme, and then labeled digoxigenin (DIG) by in vitro transcription using digoxigenin (DIG) RNA labeling kit (Roche Diagnostics, Indianapolis, IN, USA).

6. Establishment of Lrig1 knockdown cell lines

Three kinds of shRNA lentiviral vectors for Lrig1 (V2LHS_332387, V2LHS_229246 and V2LHS_404471) were designed and synthesized using GIPZ lentiviral shRNA vector. (Open Biosystems, Huntsville, AL, USA). Recombinant lentiviruses were produced by transfection of 293T cells with the packaging plasmids PMD2G and psPAX2, using CalPhos™ Mammalian Transfection Kit (Clontech, Mountain View, CA, USA) according to manufacturer′s protocol. Knockdown of Lrig1 expression was established by recombinant lentivirus infection. MKN28 and SNU638 cells (2 × 10^5cells/well) were replaced with viral supernatant with a polybrene mixture,

the cells were incubated overnight. The next day, lentivirus was removed and replaced with fresh medium and stable clones expressing shRNA were selected via further incubation with puromycin (1 μg/mL).

7. Cell proliferation assay

Established stable cell lines were seeded into 6-well culture dishes at a density of 1 x 10^5 cells per well in 2 ml of medium containing 10% FBS. Cells were trypsinized and the numbers of viable cells were counted by automatic cell counter, EVE (Nanoentek, Seoul, Korea). Samples containing trypsinized cells were mixed with 0.4% trypan blue and the mixtures were loaded on cell counting slide. The counting was performed daily throughout the 5-days. The growth curve of each group was plotted on the basis of cells number each day.

8. Statistical analysis

Statistical analyses were performed with JMP software. Paired t-tests compared the results from the control. Data are presented as average values ± SE. The results were considered significant at $P< 0.5$.

III. RESULTS

1. Various expressions of Lrig1 and EGFR in gastric and colon cancer cell lines.

Lrig1 has been reported as intestinal stem cell markers and plays a potential role in maintaining intestinal homeostasis. Homeostasis within the stem cell region is regulated by extrinsic and intrinsic signal pathways. EGFR signaling is crucial in maintaining of the intestinal epithelium. To investigate the expression of Lrig1 in gastrointestinal cell lines, I compared expression of Lrig1 in colon and gastric cancer cell lines. Lrig1 and EGFR protein expression was detected by western blotting in four types of colon cancer cell lines and five different gastric cancer cell lines (Fig. 1). The expression of Lrig1 in colon cancer cell lines was relatively constant, but was diverse in gastric cancer cell lines. Previous studies have revealed that Lrig1 acts as a negative regulator of EGFR and downregulates all four family members of ERBB receptor family. To investigate whether Lrig1 expression is in contrast to EGFR, expression of EGFR was detected. Relatively high level of EGFR expression was detected in all gastric cancer cell lines. However, out of four different colon cancer cell lines tested, only Caco2 cell line was positive for EGFR expression and no signal was detected in other cell lines (Fig.1).

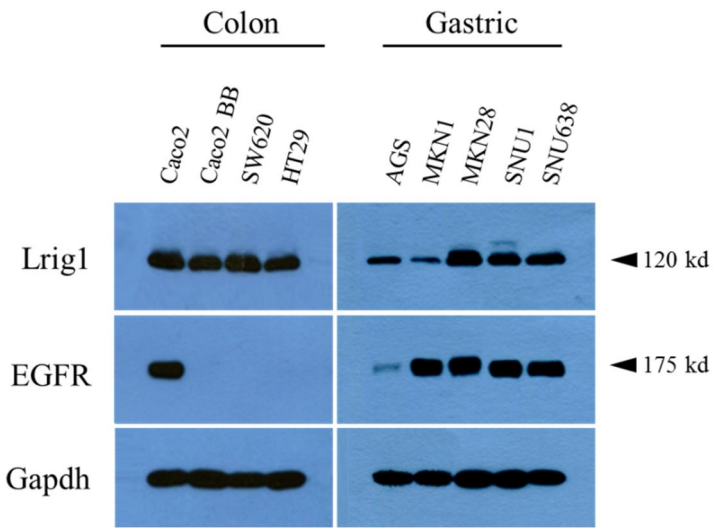

Figure 1. Expression of Lrig1 and EGFR in colon and gastric cancer cell lines. Expression of Lrig1 and EGFR was detected by western blotting in four types of colon and five types of gastric cancer cell lines. Gapdh is used a loading control.

2. Correlation between the expression of Lrig1 and EGFR in gastric cancer cell lines.

To examine the correlation between expression of Lrig1 and EGFR in protein and mRNA levels in gastric cancer cell lines, I performed western blot (Fig. 2A) and quantitative RT-PCR (Fig. 2B). All five types of gastric cancer cell lines expressed different amounts of EGFR expression. It was generally high in all except in AGS cells at protein level. The band intensity was quantified with Image J (Fig. 2A). I also observed that when Lrig1 expression was low, EGFRwas relatively high, indicating an inverse relationship between the two. Similar to protein expression, Lrig1 and EGFR mRNA level showed contrary expression pattern (Fig. 2B). These data suggest that Lrig1 may be a negative regulator of EGFR in stomach.

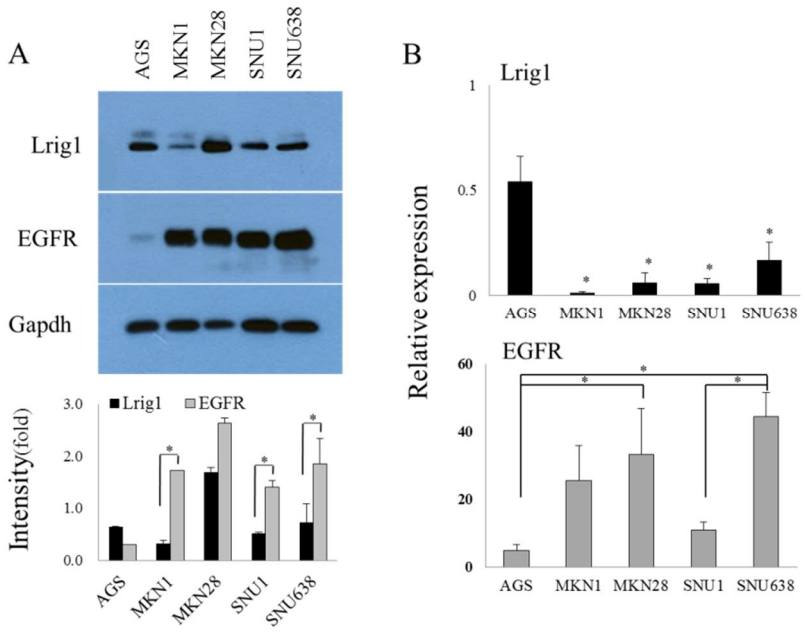

Figure 2.The differential expression of Lrig1 and EGFR in gastric cancer cell lines. Expression of Lrig1 and EGFR in gastric cancer cell lines was determined by western blotting (A) and quantitative RT-PCR (B). Band intensity quantification was measured using Image J.*, $p<0.5$

3. The cellular location of Lrig1 in gastrointestinal epithelium of normal mouse.

Next, I wondered whether Lrig1-positive cells are located within the stem cell zone in the normal mouse tissue. I confirmed the cellular localization of Lrig1 in normal mouse gastric mucosa and intestinal epithelium by *in situ* hybridization (Fig. 3). I observed Lrig1-positive cells at the isthmus region of the fundus (Fig. 3A) and antral gland of the antrum region of the stomach (Fig. 3B). In intestine, I found Lrig1-positive cells at the bottom of the crypt in the small intestine (Fig. 3C) and colon (Fig. 3D) known as stem cell zone.

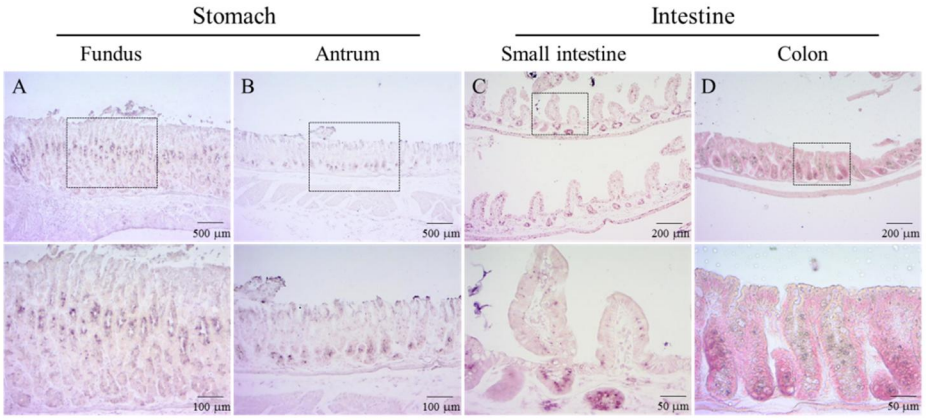

Figure 3. The expression of Lrig1 in normal mouse tissue by *in situ* hybridization. The cellular localization of Lrig1 was detected by *in situ* hybridization in the fundus (A) and the antrum (B) of mouse stomach, small intestine (C) and colon (D). Boxed areas depict regions enlarged.

4. The increased expression of Lrig1 in pre-neo metaplastic region in the mouse stomach.

I examined whether induction of metaplasia would cause changes in expression levels of Lrig1 in gastric mucosa.DMP-777 induces acute oxyntic atrophy and leads to spasmolytic polypeptide-expressing metaplasia (SPEM)without inflammation in mouse gastric mucosa.[9] To demonstrate that alteration of Lrig1 expression is dependent on metaplasia of gastric mucosa, I performed *in situ* hybridization using mouse SPEM model treated with DMP-777 for 0 day, 7 days, 14 days, and cessation after 14 days (Fig.4A). In wild type mouse, which was treated with DMP-777 for 0 day, relatively low level of Lrig1 positive cells were observed in the isthmus region in the fundus (Fig.4a) and antral gland in the antrum (Fig. 4a') of the gastric mucosa. After treatment of DMP-777 for 7 days, I could observe that Lrig1-positive cells started to increase significantly along the gland in metaplasia region and kept on increasing even after 14 days of DMP-777 treatment (Fig.4b and 4c). The

interesting thing was that when DMP-777 was stopped for 14 days and the gastric mucosa was left to recovery, the expression of Lrig1 was back to normal level (Fig. 4d). However, I observed no significant changes in the antrum (Fig. 4b', c', d'). To examine whether the increased Lrig1 positive signal resides within gastric stem cells when metaplasia is induced, I performed *in situ* hybridization using DIG-labeled Lrig1 probe which was detected by rhodamin-labeled anti-DIG IgG. And then Ki 67, known as a proliferative cell marker was fluoscent immunostained (Green) (Fig. 4B). From dual staining of Lrig1 and Ki67, it was confirmed that Ki67 was detected in Lrig1 positive cells, confirming that these cells are proliferative cells. Next, to identify whether the region where the enhanced Lrig1 signal was detected is pre-neo metaplasia region, I performed immune-histochemical staining using TFF2 antibody which is a marker of spasmolytic polypeptide-expressing metaplasia (SPEM). The results showed that TFF2 (Brown) and Lrig1 (Blue) double positive cells were expressed along the gland of DMP-777 treated mouse.

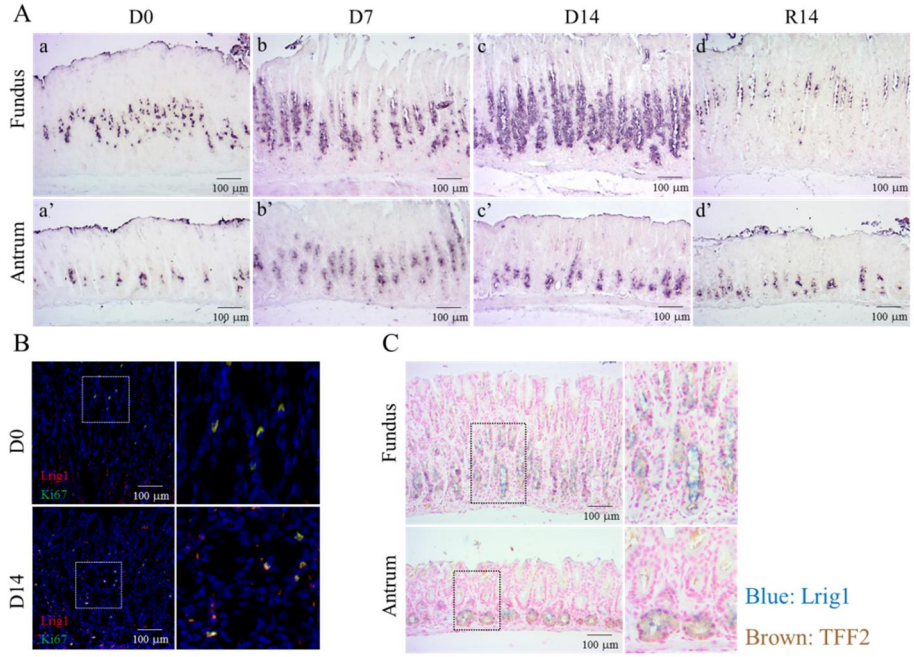

Figure 4. The alternative expression of Lrig1 in mouse SPEM model treated with DMP-777. (A) The expression of Lrig1 was detected in metaplasia region of the gastric fundic mucosa by in situ hybridization using DIG-labeled Lrig1 probe. D0 is the untreated mouse. D7 and D14 are the DMP-777 treated mouse for 7 or 14 days. R14 is the mouse with treatment of DMP-777 for 14 days and recovery for 14 days. (B) After in situ hybridization using DIG-labeled Lrig1 probe, fluorescent-immunostaining was performed with antibodies against Ki67 (Green). Sections are the fundic mucosa from DMP-777 treated mice for 0 or 14 days. (C) *In situ* hybridization for Lrig1 (Blue) and Immnohistochemistry for TFF2 (Brown), the marker of spasmolytic polypeptide-expressing metaplasia in metaplastic stomachs from DMP-777 treated mouse for 14 days. Boxed areas depict regions enlarged.

5. The alternative expression of Lrig1 during human gastric carcinogenesis.

To evaluate this change of expression of Lrig1 during human gastric carcinogenesis, I performed *in situ* hybridization using specimens of gastric cancer patient including region of normal, SPEM, intestinal metaplasia, and cancer (Fig.5). The results showed similar expression pattern with mouse study. In human normal gastric tissue, Lrig1-positive cells were detected at the isthmus of gastric mucosa. However, expression of Lrig1 showed dynamic alteration during human gastric cancer development. Compared with normal gastric tissue, Lrig1-positive cells significantly increased in SPEM region. However, upregulated Lrig1 expression was decreased in IM region and was not detected in cancer tissue. To check this change of Lrig1 in protein level, I performed immunohistochemistry with human gastric patient tissues (Fig.6).In parallel to *in situ* hybridization, Lrig1 immunoreactivity was strong in SPEM region but was hardly detected in IM region. Interestingly, the expression of Lrig1 was lost in cancer tissue. In order to confirm its role as EGFR negative regulator, EGFR expression was detected with same specimens. In SPEM region, expression of EGFR started to appear slightly and EGFR immunoreactivity in IM was detected stronger than that of SPEM. EGFR immunoreactivity was very strong in cancer region as known.

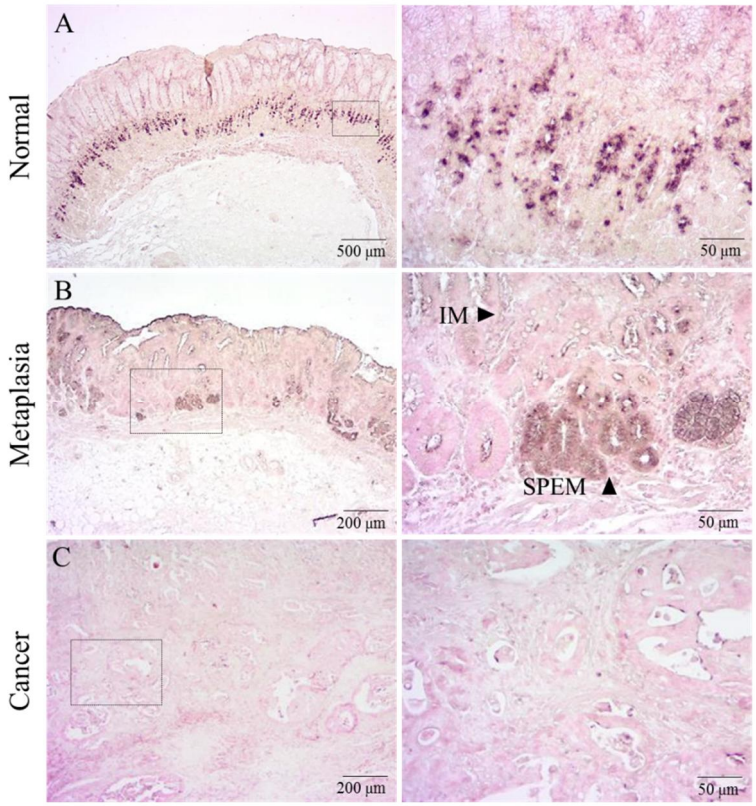

Figure 5.Lrig1 expression in human gastric normal/pre-neoplastic tissue by *in situ* hybridization. The expression of Lrig1 was detected by DIG-labeled Lrig1 probe in human gastric normal (A), pre-neoplastic (B) and cancer (C) tissue. The expression of Lrig1 was significantly increased in SPEM region but no detected in IM region. Nuclei were stained with nuclear fast red. Boxed areas depict regions enlarged.

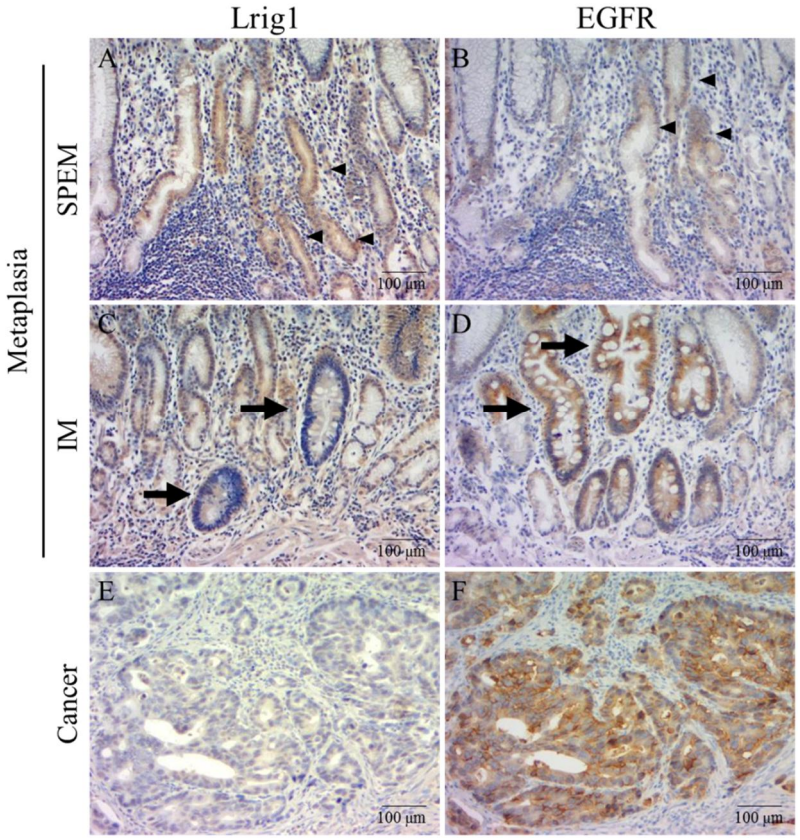

Figure 6.Immunohistochemical analysis of Lrig1 and EGFR in human gastric tissue. In SPEM region, Lrig1 was expressed strongly while EGFR started to appear slightly along with SPEM glands (A and B, arrowheads). Upregulated Lrig1 was decreased in IM region (C, arrows) and was lost in cancer tissue (E). The expression of EGFR was strongly detected in IM region (D, arrows) and cancer tissue (F).

6. The effect of Lrig1 knockdown on cell proliferation.

To investigate the role of Lrig1 in proliferative activity of gastric cancer cells, I generated stable knockdown cell lines of Lrig1 in MKN28 and

SNU638 by shRNA(Fig.7B). Out of three kinds of shRNAs, one was designed to target cytoplasmic tails of the intracellular domain, and the other two shRNAs were targeted in 3'UTR-region (Fig 7A). Because the efficiency of each shRNA was very insignificant, each shRNA lentivirus was combined in a ratio of 1:1:1 for further transduction. After establishing stable cell lines, cell growth rate was assessed by counting the cells for 5 days. As shown in Fig. 7C, Lrig1 knockdown in gastric cancer cells obviously increased the cell growth compared to control vector infected cells in both MKN28 and SNU638. These results revealed that loss of Lrig1 promotes cell growth.

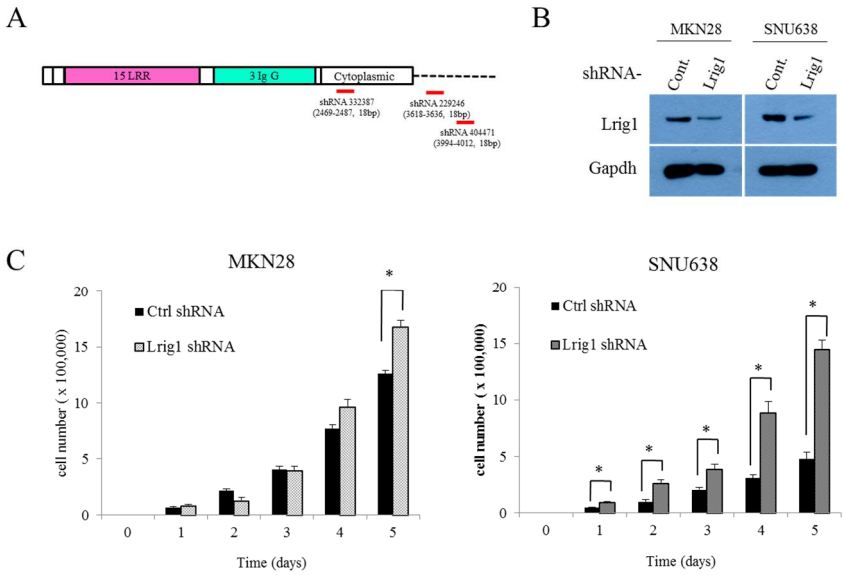

Figure 7. Generation of stable knockdown cell lines of Lrig1.(A) Structure of the Lrig1 and location of 3 kinds of shRNAs targeting sequence. (B) Lrig1 protein was detected by western blot analysis in Lrig1 knockdown stable cell lines in MKN28 and SNU638. (C) Knockdown of Lrig1 promoted cell proliferation in stable Lrig1 knockdown cell lines compare to control cell lines. *, $p<0.5$.

IV. DISCUSSION

Lrig1 is identified as quiescent intestinal stem cell marker and tumor suppressor. The cloning of Lrig1 in mouse brain[23] and human[24] has urged emerging studies to prove the importance of Lrig1 in cancer suppression as well as in cancer development. Lrig1 is also known as negative regulator of EGFR, and the interaction between Lrig1 and EGFR is thought to occur via their ectodomain. Goldoni et al showed that a soluble ectodomain of Lrig1, containing 15 LRR domains, could modulate EGFR signaling and growth promoting activity. [10,25] In addition, Lrig1 expressions are downregulated in several cancers. These indicate Lrig1as a tumor suppressor in maintaining epithelial homeostasis. Although several investigations have suggested the roles of Lrig1 in epithelial tissues, little remains known about function of Lrig1 in gastric mucosa. Here, I observed different expression pattern of Lrig1 and EGFR in gastrointestinal cancer cell lines (Fig. 1). In gastric cancer cell lines, expression of Lrig1 and EGFR was opposite of each other in protein and mRNA levels (Fig. 2A and Fig. 2B). This is consistent with the function of Lrig1in maintaining of epithelium homeostasis.

In the present studies, the expression of Lrig1, known as EGFR negative regulator, was dynamically altered during gastric carcinogenesis. Lrig1 is normally expressed in the stem cell region. In normal mouse intestine, Lrig1 was detected at the bottom of the crypt where stem cells are assumed to reside (Fig. 3A and Fig. 3B).[26] Stem cells in mouse stomach are distributed differently according to anatomically distinct areas. From our findings, it was supported that Lrig1 positive cells were located at the isthmus region in gastric fundic mucosa and at the antral gland of the antrum (Fig. 3C and 3D).[26] In addition, I observed Lrig1 positive cells in mouse SPEM model

induced by DMP-777 treatment on time-dependent manner (Fig. 4). Lrig1-positive cells were expressed differentially and its expression was enhanced in mouse metaplasia model. The most interesting thing was that the expression of Lrig1 increased in SPEM lesion whereas it was lost in IM and gastric cancer lesion. The increased expression of Lrig1 was back to normal level when DMP-777 was withdrawn. However, I found no significant altered expression in the antrum.

Gastric carcinogenesis undergoes sequence of events from chronic gastritis proceeding to gastric atrophy, intestinal metaplasia, and dysplasia. [27-29] There are two metaplastic lineages in the stomach: spasmolytic polypeptide-expressing metaplasia (SPEM) and intestinal metaplasia (IM). Generally, SPEM results from loss of parietal cells. Of note, SPEM give rise to IM. However, the relationship between SPEM and IM has long been unclear. According to the recent investigation, a single origin of dysplasia from metaplasia has been demonstrated and that metaplastic and dysplastic glands were able to expand from the same clone.[30] It would be interesting to find out whether such a genetic relation exists for SPEM and dysplasia. Furthermore, investigation on Lrig1 as a possible molecule regarding genetic modulation of SPEM progression would shed new light on its role as tumor suppressor as well.

I sought to demonstrate whether Lrig1 is co-positive with Ki-67, proliferative cell marker. The results showed that Lrig1 co-localized with Ki-67 in the SPEM region of the fundus. These indicate that when SPEM is induced in mouse stomach, proliferated progenitor cells express Lrig1. This is identical with that Lrig1 marks quiescent stem cell and associates with epithelial homeostasis following acute injury. Via dual staining for Lrig1 and TFF2, a marker of SPEM, I confirmed that Lrig1 was increased in induced SPEM region.

In human cancer tissue, I observed that Lrig1 was lost while EGFR was significantly strengthened. To examine a potential role of Lrig1 as stem cell marker and tumor suppressor in gastric mucosa, I established Lrig1 knockdowned cell lines and analyzed the growth rates. The results showed that the cell growth was significantly increased in Lrig1 knockdowned cell compared to control vector infected cell although the rate of increase was different among the cell lines. These results indicate that Lrig1 inhibited cell growth and has a potential role of tumor suppressor in gastric mucosa.

In summary, I demonstrated that there were significant correlation between Lrig1 and EGFR during gastric carcinogenesis. Here, I first report the dynamic alteration of Lrig1 expression in pre-neo metaplastic region. Its expression markedly increased in SPEM which is one of the pre-neo metaplasia. In addition, I detected complete loss of Lrig1 in gastric cancer tissue by *in situ* hybridization and immunohistochemistry. Altogether, these findings suggest that the altered expression of Lrig1 may serve as a critical point in metaplastic progression.

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V. CONCLUSION

In our study, the expression of Lrig1, known as a negative regulator of EGFR, was significantly increased during gastric carcinogenesis. The expression of Lrig1 was examined in translational and transcriptional level by *in situ* hybridization and immunohistochemistry. Its expression significantly increased in spasmolytic polypeptide-expressing metaplasia (SPEM) but I detected complete loss of Lrig1 in intestinal metaplasia (IM) and gastric cancer tissue. Additionally, I observed that knockdown of Lrig1 promoted cell growth in gastric cancer cell lines. Altogether, Lrig1 is a potential tumor suppressor in gastric mucosa, the altered expression of Lrig1 may act a critical point in metaplastic progression.

REFERENCES

1. Bray F, Ferlay J, Laversanne M, Brewster DH, Gombe Mbalawa C, Kohler B, et al. Cancer Incidence in Five Continents: Inclusion criteria, highlights from Volume X and the global status of cancer registration. *International journal of cancer.* Nov 1 2015;137(9):2060-2071.
2. Goldenring JR, Nomura S. Differentiation of the gastric mucosa III. Animal models of oxyntic atrophy and metaplasia. *American journal of physiology. Gastrointestinal and liver physiology.* Dec 2006;291(6):G999-1004.
3. Blaser MJ, Parsonnet J. Parasitism by the "slow" bacterium Helicobacter pylori leads to altered gastric homeostasis and neoplasia. *The Journal of clinical investigation.* Jul 1994;94(1):4-8.
4. Goldenring JR, Nam KT. Oxyntic atrophy, metaplasia, and gastric cancer. *Progress in molecular biology and translational science.* 2010;96:117-131.
5. Weis VG, Goldenring JR. Current understanding of SPEM and its standing in the preneoplastic process. *Gastric cancer : official journal of the International Gastric Cancer Association and the Japanese Gastric Cancer Association.* 2009;12(4):189-197.

6. Lee HJ, Nam KT, Park HS, Kim MA, Lafleur BJ, Aburatani H, et al. Gene expression profiling of metaplastic lineages identifies CDH17 as a prognostic marker in early stage gastric cancer. *Gastroenterology.* Jul 2010;139(1):213-225 e213.

7. Choi E, Hendley AM, Bailey JM, Leach SD, Goldenring JR. Expression of Activated Ras in Gastric Chief Cells of Mice Leads to the Full Spectrum of Metaplastic Lineage Transitions. *Gastroenterology.* Apr 2016;150(4):918-930 e913.

8. Fox JG, Li X, Cahill RJ, Andrutis K, Rustgi AK, Odze R, et al. Hypertrophic gastropathy in Helicobacter felis-infected wild-type C57BL/6 mice and p53 hemizygous transgenic mice. *Gastroenterology.* Jan 1996;110(1):155-166.

9. 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.* Dec 2010;139(6):2028-2037 e2029.

10. Segatto O, Anastasi S, Alema S. Regulation of epidermal growth factor receptor signalling by inducible feedback inhibitors. *Journal of cell science.* Jun 1 2011;124(Pt 11):1785-1793.

11. Gur G, Rubin C, Katz M, Amit I, Citri A, Nilsson J, et al. LRIG1 restricts growth factor signaling by enhancing receptor ubiquitylation and degradation. *The EMBO journal.* Aug 18 2004;23(16):3270-3281.

12. Laederich MB, Funes-Duran M, Yen L, Ingalla E, Wu X, Carraway

KL, et al. The leucine-rich repeat protein LRIG1 is a negative regulator of ErbB family receptor tyrosine kinases. *The Journal of biological chemistry.* Nov 5 2004;279(45):47050-47056.

13. Jensen KB, Watt FM. Single-cell expression profiling of human epidermal stem and transit-amplifying cells: Lrig1 is a regulator of stem cell quiescence. *Proceedings of the National Academy of Sciences of the United States of America.* Aug 8 2006;103(32):11958-11963.

14. Jensen KB, Collins CA, Nascimento E, Tan DW, Frye M, Itami S, et al. Lrig1 expression defines a distinct multipotent stem cell population in mammalian epidermis. *Cell stem cell.* May 8 2009;4(5):427-439.

15. Powell AE, Wang Y, Li Y, Poulin EJ, Means AL, Washington MK, et al. The pan-ErbB negative regulator Lrig1 is an intestinal stem cell marker that functions as a tumor suppressor. *Cell.* Mar 30 2012;149(1):146-158.

16. Suzuki Y, Miura H, Tanemura A, Kobayashi K, Kondoh G, Sano S, et al. Targeted disruption of LIG-1 gene results in psoriasiform epidermal hyperplasia. *FEBS letters.* Jun 19 2002;521(1-3):67-71.

17. Wong VW, Stange DE, Page ME, Buczacki S, Wabik A, Itami S, et al. Lrig1 controls intestinal stem-cell homeostasis by negative regulation of ErbB signalling. *Nature cell biology.* Apr 2012;14(4):401-408.

18. Thomasson M, Hedman H, Guo D, Ljungberg B, Henriksson R. LRIG1 and epidermal growth factor receptor in renal cell carcinoma: a quantitative RT--PCR and immunohistochemical analysis. *British journal of cancer.* Oct 6 2003;89(7):1285-1289.

19. !!! INVALID CITATION !!!

20. Miller JK, Shattuck DL, Ingalla EQ, Yen L, Borowsky AD, Young LJ, et al. Suppression of the negative regulator LRIG1 contributes to ErbB2 overexpression in breast cancer. *Cancer research.* Oct 15 2008;68(20):8286-8294.

21. Ljuslinder I, Golovleva I, Palmqvist R, Oberg A, Stenling R, Jonsson Y, et al. LRIG1 expression in colorectal cancer. *Acta oncologica.* 2007;46(8):1118-1122.

22. Hedman H, Henriksson R. LRIG inhibitors of growth factor signalling - double-edged swords in human cancer? *European journal of cancer.* Mar 2007;43(4):676-682.

23. Suzuki Y, Sato N, Tohyama M, Wanaka A, Takagi T. cDNA cloning of a novel membrane glycoprotein that is expressed specifically in glial cells in the mouse brain. LIG-1, a protein with leucine-rich repeats and immunoglobulin-like domains. *The Journal of biological chemistry.* Sep 13 1996;271(37):22522-22527.

24. Hedman H, Nilsson J, Guo D, Henriksson R. Is LRIG1 a tumour suppressor gene at chromosome 3p14.3? *Acta oncologica.*

2002;41(4):352-354.

25. Goldoni S, Iozzo RA, Kay P, Campbell S, McQuillan A, Agnew C, et al. A soluble ectodomain of LRIG1 inhibits cancer cell growth by attenuating basal and ligand-dependent EGFR activity. *Oncogene.* Jan 18 2007;26(3):368-381.

26. Vries RG, Huch M, Clevers H. Stem cells and cancer of the stomach and intestine. *Molecular oncology.* Oct 2010;4(5):373-384.

27. Hoffmann W. Current Status on Stem Cells and Cancers of the Gastric Epithelium. *International journal of molecular sciences.* 2015;16(8):19153-19169.

28. Fox JG, Wang TC. Inflammation, atrophy, and gastric cancer. *The Journal of clinical investigation.* Jan 2007;117(1):60-69.

29. Correa P, Houghton J. Carcinogenesis of Helicobacter pylori. *Gastroenterology.* Aug 2007;133(2):659-672.

30. Gutierrez-Gonzalez L, Graham TA, Rodriguez-Justo M, Leedham SJ, Novelli MR, Gay LJ, et al. The clonal origins of dysplasia from intestinal metaplasia in the human stomach. *Gastroenterology.* Apr 2011;140(4):1251-1260 e1251-1256.

< ABSTRACT (IN KOREAN)>

위 암 발생 과정에서 EGFR 억제 조절 인자인 Lrig1의 발현 양상에 관한 연구

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양 미 정

Lrig1 (Leucine-rich repeats and immunoglobulin-like domains 1)은 막단백질로 수용체 수준을 낮게 조절함으로써 EGFR의 신호 전달에서 길항작용을 한다. 최근 연구에서는 Lrig1은 장 줄기세포의 표지인자로 장내에서 4번자리에 위치하며 분열을 잘하지 않고 오래 생존하는 휴지상태의 세포라고 보고하였다. Lrig1은 여러가지 암에서 그 발현정도가 낮다고 보고되었고, 이 결과는 Lrig1이 암 억제 유전자로서 상피세포의 항상성 유지에 중요한 역할을 한다는 사실을 시사한다. 하지만 위에서는 Lrig1의 역할에 대해서 알려진 바가 없어, 이번 연구에서는 위암의 전암 병변 모델로 알려진DMP-777을 투여한 마우스 모델을 이용하여 암 발생 과정에서 Lrig1의 발현 변화에 대하

여 *in situ* hybridization을 통해 RNA 수준에서 분석하였고, 사람의 위암 조직을 이용하여 위암 발생 과정에서의 Lrig1의 발현 변화를 분석하였다. 암 발생 과정 중에서 Lrig1은spasmolytic polypeptide-expressing metaplasia (SPEM) 과정에서는 발현이 증가하지만 intestinal metaplasia (IM)과 암 진행 부위에서는 발현이 사라지는 것을 관찰하였다. EGFR의 발현은 각각의 metaplasia에서 Lrig1과 상보적으로 발현하였고, 위 암 조직에서 높게 발현되는 것을 관찰하였다. 위 암 부위에서 높게 발현한다. 또한 위 암 세포주 내에서 Lrig1의 발현에 따라 세포의 증식에 미치는 영향을 분석한 결과, Lrig1 knockdown 세포에서 세포 증식이 증가되는 것을 관찰하였다. 이러한 결과들로 EGFR의 억제 조절 인자인 Lrig1이 위 암 발생 과정에서 매우 중요한 역할을 할 것으로 사료된다.

핵심되는말: Lrig1, gastric carcinogenesis, SPEM, IM

PUBLICATION LIST

1. Yu S, **Yang M**, Nam KT. Mouse models of gastric carcinogenesis. J Gastric Cancer. 2014;14(2):67-86.
2. Minjeong Kim, Jun-Won Yun, Kyeho Shin, Yejin Cho, **Mijeong Yang**, Ki Taek Nam, Kyung-Min Lima, Expression levels of GABA-A receptor subunit alpha 3, *Gabra3* and lipoprotein lipase, *Lpl* are associated with the susceptibility to acetaminophen-induced hepatotoxicity. Biomolecules & Therapeutics. 2016