

Fermented kimchi rejuvenated precancerous atrophic gastritis via mitigating *Helicobacter pylori*-associated endoplasmic reticulum and oxidative stress

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Dietary intervention to prevent *Helicobacter pylori* (*H. pylori*)-gastric cancer might be ideal by long-term intervention, rejuvenating action, and no risk of bacterial resistance. Stimulated with finding that kimchi prevented *H. pylori*-gastric cancer, we compared the efficacy of cancer preventive kimchi (cpkimchi) and standard recipe kimchi (skimchi) and the efficacy between fermented kimchi and non-fermented kimchi (kimuchi) in *H. pylori*-initiated gastric cancer model and explored novel mechanisms hinted from RNAseq transcriptome analysis. Animal models assessing gastric pathology on 24 and 36 weeks after *H. pylori* initiated, salt diet-promoted gastric mutagenesis model showed fermented cpkimchi afforded the best outcome of either rejuvenating atrophic gastritis or inhibiting tumorigenesis compared to skimchi and kimuchi. Highest inhibition of atrophic gastritis was achieved with cpkimchi, while significantly lower in kimuchi. Transcriptomic analysis showed ameliorated-endoplasmic reticulum (ER) stress, -oxidative stress, and -apoptosis as major rejuvenating action of cpkimchi. Homogenates from animal model showed that elevated expressions of p-PERK, IRE, ATF6, p-elf, and XBP1 in control group, while significantly decreased with dietary intake of only cpkimchi. Significantly increased expressions of HO-1 and γ -GCS were only noted with cpkimchi. Conclusively, long-term dietary intervention of fermented cpkimchi can be potential way preventing *H. pylori*-associated carcinogenesis via rejuvenation of atrophic gastritis.

Key Words: *H. pylori*, dietary intervention, cpkimchi, ER stress, cancer prevention

Though *Helicobacter pylori* (*H. pylori*) infection had been defined as class I carcinogen for gastric cancer and had been associated with diverse gastric diseases as well as extra-gastric diseases, the bacterium frequently persists in the human host without inducing disease and plays a beneficial role in health in some cases.⁽¹⁾ However, as efforts to decrease gastric cancer mortality, general eradication strategy had been performed in Japan, but we should await long-term outcome. As another intervention for similar purpose, non-microbial intervention had been tried. Among these, as far as gastric carcinogenesis is concerned, dietary factors themselves are dual-edged swords, that is, implicated in carcinogenesis, but possibly preventive on other hand. For example, salty food and red/processed meat intakes were proven to be associated with an increased

risk of gastric non-cardia cancer, whereas vegetable and fruits are protective factors especially in *H. pylori* antibody-positive subject.⁽²⁾ Although the cohort-based evidence is still lacking, a few dietary approach with antioxidants or nutraceuticals are available to prevent *H. pylori*-associated gastric diseases.

Kimchi is a traditional Korean fermented side-dish, especially probiotic food because red peppers and other condiments have undergone lactic acid fermentation, in addition to profuse levels of several phytochemicals enriched in each gradient of kimchi, vitamins, minerals, and dietary fibers. A case-control study to assess the influence of kimchi on gastric cancer showed that kimchi significantly decreased the risk of gastric cancer.⁽³⁾ In a follow up study, they also found that gastric cancer risk could be decreased if those with *H. pylori* infection increase their intakes of antioxidant vitamins.⁽⁴⁾ Just like yogurt and other fermented milk products had been important sources for probiotics in Western country, kimchi can be defined as “probiotic food” in Korean because it contains myriad types of probiotic lactobacillus, *L. plantarum* as a representative probiotic strain.^(5,6) *L. plantarum* isolated from kimchi exerted significant anti-inflammatory actions against *H. pylori* infection.⁽⁷⁾ Though salts are contained in kimchi, the recent trend to reduce salt concentration lesser than 2.0–2.2%, well-designed clinical and Cohort study consistently reveal that kimchi is cancer preventive fermented foods.

As a dietary intervention for *H. pylori* infection, we invented novel cancer preventive kimchi (cpkimchi) recipe and put hypothesis that dietary intervention of cpkimchi can prevent *H. pylori*-associated gastric cancer in mice model. Generation of cpkimchi was based on the addition of mustard leaf, pear, mushroom, Chinese pepper, and sea tangle juice onto standardized kimchi recipe (skimchi) chosen by many previous studies. As results, long-term intervention of cpkimchi showed significant preventive effects of *H. pylori*-induced gastric cancer either through the rejuvenation of *H. pylori*-associated atrophic gastritis or through significant control of gastric tumorigenesis. Stimulated with this outcome, in the current study, we aimed to compare the efficacy of current cpkimchi with other formula of kimchi, skimchi and non-fermented Japanese style kimchi (kimuchi) on these ameliorating actions. Coinciding with these

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works, we also performed RNA sequence analysis (RNAseq) to discover the major contributing actions of cpkimchi under *H. pylori* infection and documented the biological actions discovered with RNAseq analysis. In this study, we tried to validate the major biological actions in above animal model.

Material and Methods

Preparation of skimchi, cpkimchi, and non-fermented kimchi (kimuchi). Preparation of skimchi was based on the standardized kimchi recipe of CJ Food Research Institute (CJ Food Blossom Park, Suwon, Korea). The skimchi is made of brined Korean cabbage, red pepper powders, garlic, ginger, anchovy juice, redish, green onion, glutinous rice paste based on folk recipe. In addition to these ingredients, cpkimchi production was based on additional supplements such as onion, mustard leaf, pear, sea tangle juice. *Leuconostoc mesenteroides* CJ LM119, *Lactobacillus plantarum* CJ LP133 were included in cpkimchi to facilitate fermentation. Kimuchi, was purchased on the domestic market in Tokyo, Japan. Commonly, we reduced salt intake lesser than 1.8%, salts are used in making binned baechu cabbage (a kind of Chinese cabbage) and some anchovy.

Kimchi processing used for *in vitro* cell experiment and *in vivo* animal model. All of the kimchi samples were lyophilized and freeze-dried kimchi samples underwent an extraction process with 20 times of methanol by stirring overnight. Finally, the kimchi methanol extracts were concentrated by heat evaporation (Eyela rotary evaporator system) and stored at 4°C. Since the usual serving dose of kimchi in Korea is approximately 100 g/day upon individual taste, the extracted cpkimchi was mixed into diet pellet, changed daily, in two serving dose for animal, 1.7 g/kg/day and 5.1 g/kg/day equivalent with usual general intake dose of kimchi in Korean. The skimchi, cpkimchi and kimuchi extracts for *in vitro* experiment was dissolved into 5 mg/ml and 10 mg/ml in order to execute *in vitro*-cell experiment.

***H. pylori* culture.** *H. pylori* strain ATCC43504 (American Type Culture Collection, a *cagA*⁺ and *vacA* s1-m1 type's strain) was used for *in vitro* model and Sydney strain (SS1, a *cagA*⁺, *vacA* s2-m2 strain adapted for mice infection) for *in vivo* model. *H. pylori* were cultured at 37°C in BBL Trypticase soy (TS) agar plate with 5% sheep blood (TS/II; BD Biosciences, Franklin Lakes, NJ) under microaerophilic condition (BD GasPak EZ Gas Generating Systems, BD Biosciences) for 3 days. The bacteria were harvested in clean TS broth, centrifuged at 3,000 × *g* for 5 min, and resuspended in the broth at a final concentration of 1 × 10⁹ colony-forming units (CFUs)/ml. In all experiments, cultures grown for 72 h on TS agar plates were used.

Animal experimental procedure. Five-week-old C57BL/6 mice (Charles River, Tokyo, Japan) were fed sterilized commercial pellet diets (Biogenomics, South Korea) and sterile water *ad libitum*, and housed in an air-conditioned biohazard room at a temperature of 24°C. After 1 week of adaptation, 20 mg/kg pantoprazole was injected three times to facilitate *H. pylori* colonization through lowered gastric acidity. And then each animal was intragastrically inoculated with a suspension of *H. pylori* containing 1 × 10⁹ CFUs/ml with an equal volume (0.3 ml) of clean TS broth using gastric intubation needles. All group were given injections of *H. pylori* total fourth times within a week. One group of 10 mice (uninfected group) was given injections of clean TS broth. The mice were fed a special pellet diet based on AIN76 containing 7.5% NaCl to generate more exacerbated data for 4 weeks. Then, *H. pylori* positive mice were randomly divided into five groups (*n* = 10, *H. pylori* control group = 20). Pellet diet AIN76 containing 7.5% NaCl were administrated for 24 weeks and 36 weeks to promote *H. pylori*-induced carcinogenic process in all infected animals.⁽⁸⁾ Experimental groups are shown in Fig. 1A and Fig. 3A,

respectively and all animal studies were carried out in accordance with protocols approved by the Institutional Animal Care and Use Committee (IACUC) of CHA University CHA Cancer Institute after IRB approval (Approve number #2016-0501). Stomachs were isolated and subjected to further histologic examination, Western blotting, and RT-PCR.

Gross index. After sacrificing the animals, the isolated stomachs were cut open along the greater curvature and washed in ice-cold saline. To investigate the degree of gross mucosal damage, the mucosal sides of the stomachs were photographed using a digital camera and part of the mucosa was immediately fixed with 10% formalin solution. The gross damage of the gastric mucosa was assessed by three gastroenterologists, who were blinded to the treatments, using a gross ulcer index.⁽⁹⁾ These lesions types were defined as follows; type I, presence of edema, hyperemia, or single submucosal punctiform hemorrhage; type II, presence of submucosal hemorrhagic lesions with small erosions; type III, presence of deep ulcer with erosions and invasive lesions.

Histopathology. For histopathological analysis, the stomach was fixed in 10% neutralized buffered formalin, processing using the standard method and embedded in paraffin. Sections of 4 μm thickness were then stained with hematoxylin and eosin (H&E).⁽¹⁰⁾ The glandular mucosae of corpus and antrum were examined histologically. The pathological changes of *H. pylori*-infection, such as inflammatory cells infiltration, erosive lesions, ulceration, dysplasia, adenoma formation (precancerous lesion), were graded by three gastroenterologists, who were blinded to the treatments, using an index of histologic injury defined.⁽¹¹⁾ In this study, inflammation was defined as grade the infiltration of inflammatory cells, 0: none, 1: under the lamina propria, 2: half of mucosa 3: until the epithelial gland layer (all mucosa). The erosion was defined as proportion of erosive lesion, 0: none, 1: loss of epithelial gland layer (1/3 proportion), 2: two-third portion of mucosa (2/3 proportion) 3: all mucosa (3/3 proportion).

Western blot analysis. Extracted stomach tissues were washed twice with PBS and then lysed in ice-cold cell lysis buffer (Cell Signaling Technology, Denver, MA) containing 1 mM phenylmethylsulfonyl fluoride (PMSF, Sigma Aldrich, St. Louis, MO). After 20 min of incubation, samples were centrifuged at 10,000 × *g* for 10 min. Supernatants were then collected. Proteins in lysates were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes, which were incubated with primary antibodies, washed, incubated with peroxidase-conjugated secondary antibodies, rewashed, and then visualized using an enhanced chemiluminescence (ECL) system (GE Healthcare, Buckinghamshire, UK).

***In vitro H. pylori*-infected cell model.** Gastric adenocarcinoma cell line, AGS cell, was purchased from ATCC (Manassas, VA), where the cells were properly stored and routinely authenticated. After resuscitation in our lab, all the cells were used no longer than 6 months. AGS cells were cultured in RPMI-1640 medium (Gibco BRL, Gaithersburg, MD) and the mediums supplemented with 10% fetal bovine serum (Gibco BRL) at 37°C in 5% CO₂. The cells were plated into 6-well plates at 10⁵ cells/ml and allowed to adhere for 24 h. And the 50 multiplicity of infection (MOI) *H. pylori* exposed to cells and cpkimchi extract was applied in the test wells at 5 or 10 mg/ml concentrations for 6 h.

RNA isolation and reverse transcription-polymerase chain reaction. After treatment, the medium was removed by suction, and cells were washed with Dulbecco's PBS twice. RiboEX (500 μl; GeneAll, Seoul, Korea) was added to plates that were incubated for 10 min at 4°C. RiboEX was harvested and placed in a 1.5-ml tube, and 100 μl of chloroform was added and gently mixed. After incubation for 10 min in ice, samples

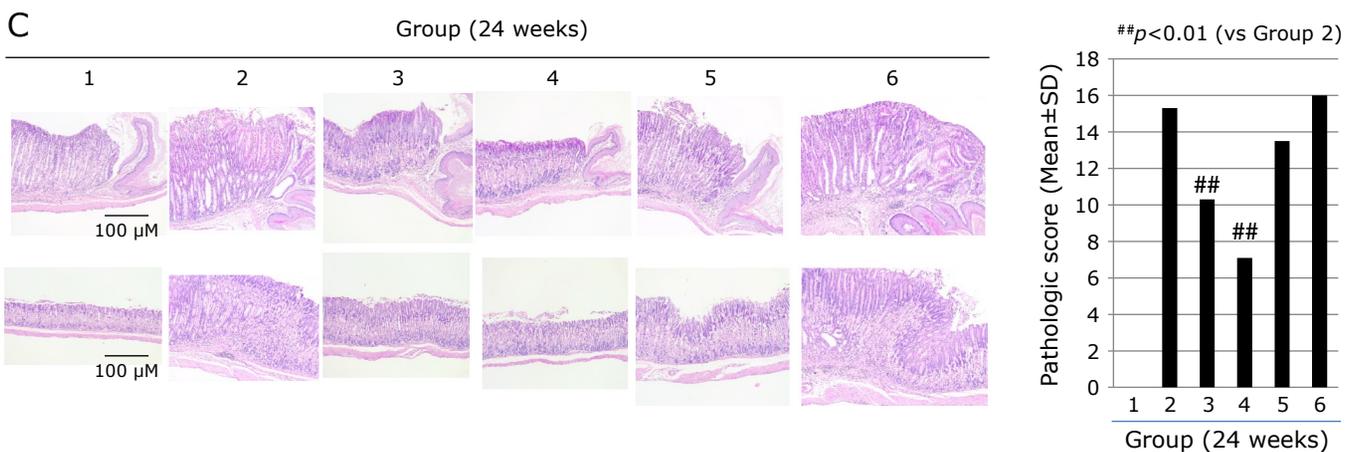
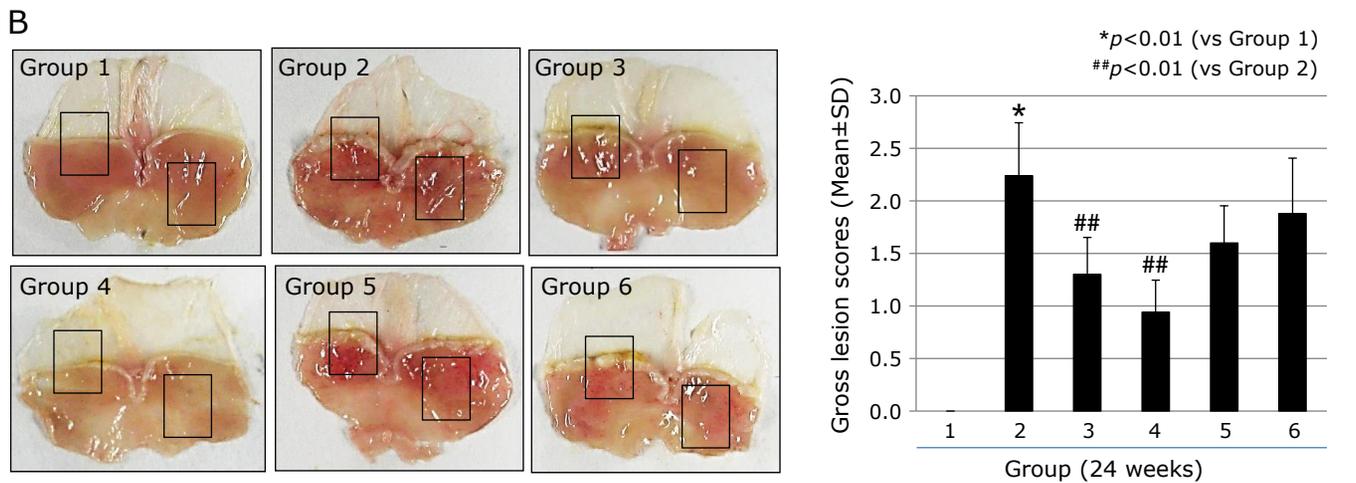
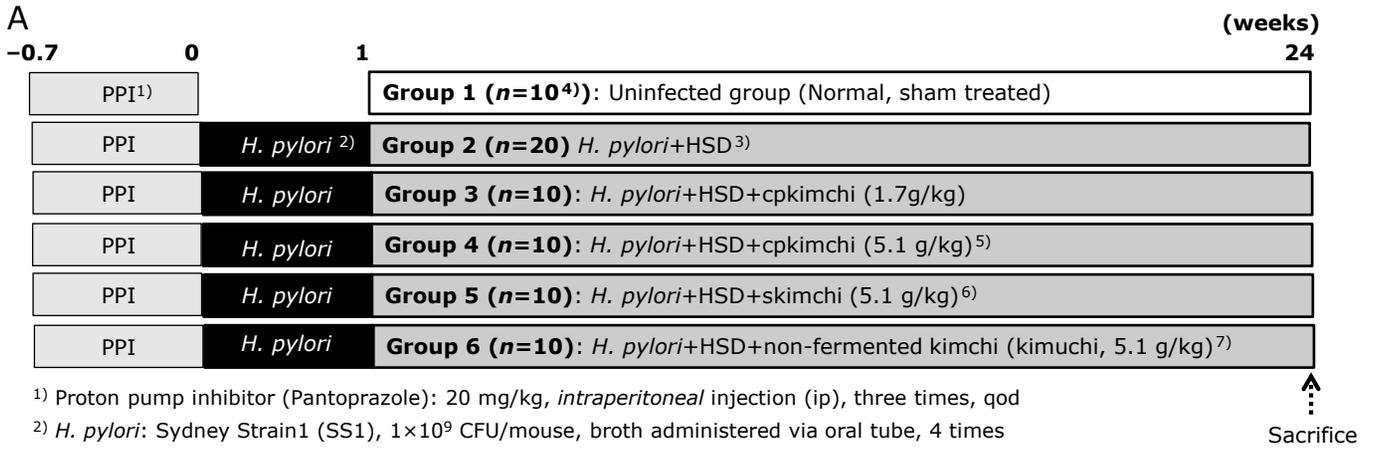


Fig. 1. Dietary intake of three kinds of kimchi, fermented standard recipe kimchi (skimchi), fermented special recipe cancer preventive kimchi (cpkimchi), and non-fermented Japanese style kimchi (kimuchi), on *H. pylori* initiated-, high salt diet-promoted- CAG, 24 weeks model. (A) Scheme for group, Group 1 as normal control, Group 2 as *H. pylori*-associated CAG control, Group 3 as disease control treated with 1.7 g/kg cpkimchi, Group 4 as disease control treated with 5.1 g/kg cpkimchi, Group 5 as disease control treated with 5.1 g/kg skimchi, and Group 6 as disease control treated with 5.1 g/kg kimuchi. Mice were sacrificed after 24 weeks of *H. pylori* infection. Kimchi was administered as diet pellet containing manner. (B) Representative gross photo of resected stomach according to group and mean gross lesion scores according to group. (C) Representational pathology and mean pathological scores according to group, ×40 magnification. See color figure in the on-line version.

were centrifuged at $10,000 \times g$ for 30 min. Supernatants were extracted and mixed with 200 μ l of isopropanol, and mixtures were incubated at 4°C for 1 h. After centrifuging at $13,000 \times g$ for 30 min, the pellet was washed with 70% (v/v) ethanol. After allowing the ethanol to evaporate completely, the pellet was dissolved in 100 μ l of diethylene pyrocarbonate-treated water (Invitrogen Life Technologies, Carlsbad, CA). cDNA was prepared using a reverse transcriptase originating from Murine-Moloney leukemia virus (Promega, Madison, WI), according to the manufacturer's instructions. PCR was performed using over 30 cycles of 94°C for 20 s, 58°C for 30 s, and 72°C for 45 s. Oligonucleotide primers were purchased from Bioneer (Seoul, Korea). Primers used were as follows; *Cox-2*; Forward: GGT CTG GTG CCT GGT CTG ATG ATG and Reverse: GTC CTT TCA AGG AGA ATG GTG C, *IL-1 β* ; Forward: TCC AGG ATG AGG ACA TGA GCA C and Reverse: GAA CGT CAC ACA CCA GCA GGT TA, *IL-6*; Forward: CCG GAG AGG AGA CTT CAC AG and Reverse: TCC ACG ATT TCC CAG AGA AC, *IFN- γ* ; Forward: ACA ATG AAC GCT ACA CAC TG and Reverse: TCA AAC TTG GCA ATA CTC AT, *VEGF*; Forward: GAA GCT ACT GCC GTC CGA TT and Reverse: TCC TCT TCC TTC ATG TCA GGC, *PDGF*; Forward: ACG TCA TGT TAC GGC TTC CT and Reverse: CAG TGT GAC TGT GTC TCC CC, *iNOS*; Forward: GGC CTC TCA GCT CAC CCC GA and Reverse: CCA GGC GCA CTG TCT GGT GG, *RANTES*; Forward: GAA GAT CTC TGC AGC TGC CCT and Reverse: GCT CAT CTC CAA ATA GTT GA, *VCAM1*; Forward: CCT CAC TTG CAG CAC TAC GGG CT and Reverse: TTT TCC AAT ATC CTC AAT GAC GGG, *ICAM1*; Forward: CCT GTT TCC TGC CTC TGA AG and Reverse: GTC TGC TGA GAC CCC TCT TG, Mouse *GAPDH*; Forward: CAG GGC AAA TTC AAC GGC ACA GTC AA and Reverse: GTT CAC ACC CAT CAC AAA CAT GG. Also, primers used for RT-PCR in human cells were as follows; *PPP1R15A*; Forward: ATC ACA CCG GGA GTG TTG TC and Reverse: CCT TCA GGA AGG CAC TTG GT, *DDIT3*; Forward: GAC ACT ACG TCG ACC CCC TA and Reverse: AGG GAC TCT CCC CAT TCC TC, *CHAC1*; Forward: GCA GGG AGA CAC CTT CCA TC and Reverse: GCC AAT GCC TTC AGT GGT TG, *FGF21*; Forward: TGT AGC TCC TGC CAA ATG GG and Reverse: GTG GAG CGA TCC ATA CAG GG, *ASNS*; Forward: GGA GCC AGG TCG GTA TAA GC and Reverse: TTC CAA ACA GCG GGT CAA CT, and *CTH*; Forward: CAC CTG GGA GTA GTG GC and Reverse: CGG ACT TCA AGC CCA TTC CT.

RNA sequencing. Library preparation and sequencing Libraries were prepared from 2 μ g of total RNA using the SMARTer Stranded RNA-Seq Kit (Clontech Laboratories, Inc.). The isolation of mRNA was performed using the Poly(A) RNA Selection Kit (LEXOGEN, Inc., Vienna, Austria). The isolated mRNAs were used for the cDNA synthesis and shearing, following manufacture's instruction. Indexing was performed using the Illumina indexes 1–12. The enrichment step was carried out using of PCR. Subsequently, libraries were checked using the Agilent 2100 bioanalyzer (DNA High Sensitivity Kit) to evaluate the mean fragment size. Quantification was performed using the library quantification kit using a StepOne Real-Time PCR System (Life Technologies). High-throughput sequencing was performed as paired-end 100 sequencing using HiSeq 2500 (Illumina). RNA sequencing (RNA-Seq) reads were mapped using TopHat software tool in order to obtain the alignment file. Differentially expressed gene (DEG) were determined based on counts from unique and multiple alignments using coverage in Bedtools (Quinlan AR). The RT (Read Count) data were processed based on Quantile normalization method using EdgeR within R (R development Core Team) using Bioconductor. The alignment files also were used for assembling transcripts, estimating their abundances and detecting differential expression of genes or isoforms using cufflinks. And we used the

FPKM (fragments per kilobase of exon per million fragments) as the method of determining the expression level of the gene regions. Gene classification was based on searches done by DAVID (<http://david.abcc.ncifcrf.gov/>).

Statistical analysis. Results are expressed as the mean \pm SD. Statistical analyses of the data were performed using Graphpad (GraphPad Software, San Diego, CA) and SPSS software (ver. 12.0, Chicago, IL). All other experiments were performed on triplicated independent occasions. The data were analyzed by one-way analysis of variance (ANOVA) tests, and the statistical significance between groups was determined by Tukey's multiple comparison test. Significance were considered at $p < 0.05$.

Results

Fermented cpkimchi, specially recipe kimchi, ameliorated *H. pylori*-induced chronic atrophic gastritis (CAG). Supported with our previous publication that specially recipe fermented kimchi, cancer preventive kimchi, efficiently prevented either *H. pylori*-associated CAG or gastric carcinogenesis,⁽¹²⁾ we conducted the current study under the additional aims that, one was to compare the efficacy between cpkimchi and skimchi, and the other was to compare between fermented kimchi and kimuchi against *H. pylori*-initiated, high salted drinking water-promoted atrophic gastritis models. Briefly, Group 3 as 1.7 g/kg cpkimchi administered for 24 weeks, Group 4 as 5.1 g/kg cpkimchi administered for 24 weeks, Group 5 as 5.1 g/kg fermented skimchi, and group 6 as 5.1 g/kg kimuchi was generated (Fig. 1A). When sacrificed at 24 weeks, as seen in Fig. 1B, Group 2 control showed edematous, erythematous, and focally swollen gastric glandular mucosa as well as scattered pale and thin gastric mucosa suggestive of atrophic gastritis (Fig. 1B and C). These gross findings were scored according to degree of erythema, degree of swelling, and degree of focal enlarged mucosa. As result, statistically significantly decreased gross lesion scores were noted in Group 3, Group 4, and Group 5 compared to Group 2, but not in Group 6, suggesting fermented kimchi all showed significant decreases in gross lesion score, while not in kimuchi ($p < 0.05$). These findings were further analyzed with pathological score as seen in Fig. 1C, pathological scores including degree of inflammation, degree of mucosal proliferation, degree of atrophic changes, and mucosal erosions/ulcers. In Group 2, the mucosal proliferation was prominent accompanied with severe inflammatory cell proliferation, decreases of gastric parietal cells, and multiple mucosal erosions. Significantly decreases in pathological lesion scores were only significantly observed in Group 3 and Group 4, while none in Group 5 and Group 6, signifying that fermented cpkimchi alone exerted significant protection from *H. pylori*-induced CAG ($p < 0.001$).

Concerted actions of fermented kimchi significantly ameliorated *H. pylori*-induced CAG. Referenced with our previous study that cancer preventive actions of cpkimchi were based on anti-inflammatory and anti-tumorigenic action,⁽¹²⁾ we measured the changes of inflammatory cytokines and angiogenic factors in this model according to group. As shown in Fig. 2A, expressions of *Cox-2*, *IL-1 β* , *IL-6*, and *IFN- γ* mRNA were all significantly increased in Group 2 ($p < 0.005$). However, remarkably elevated levels of these inflammatory mediators relevant to 24 weeks of *H. pylori* infection were significantly decreased in Group 3 and Group 4, but not in Group 6 ($p < 0.001$), suggesting significant anti-inflammatory actions were exerted with fermented cpkimchi, while not in kimuchi. Measuring angiogenic growth factors such as *VEGF* and *PDGF* mRNA, similar findings were obtained that cpkimchi significantly decreased *H. pylori*-associated angiogenic factors (Fig. 2A). 15-hydroxyprostanidin dehydrogenase (15-PGDH)

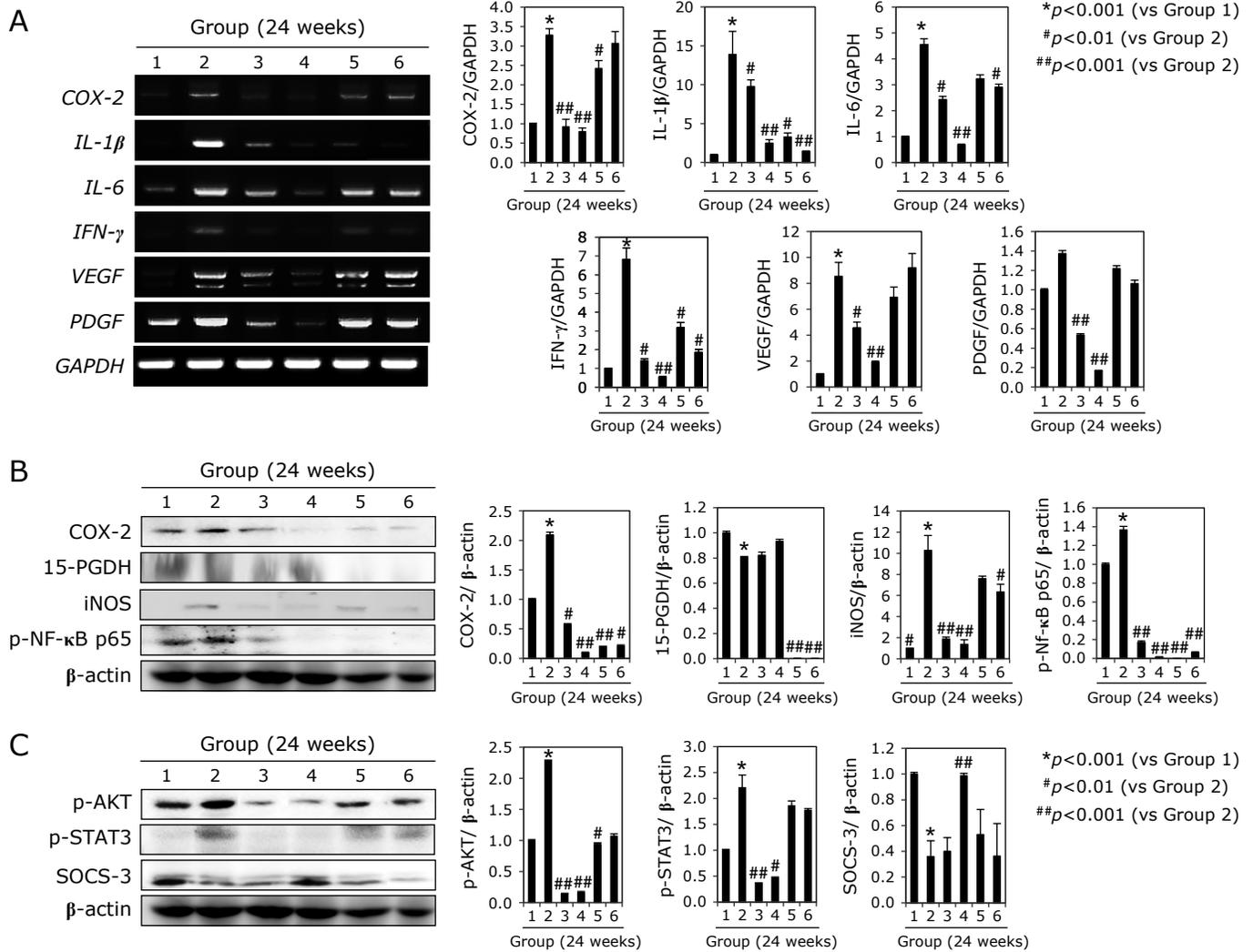


Fig. 2. Changes of inflammatory genes, NF- κ B, AKT, 15-PGDH, and STAT3 according to group (24 weeks). (A) RT-PCR for COX-2, IL-1 β , IL-6, IFN- γ , VEGF, and PDGF mRNA according to group. Mean expressions levels after triplicate measurement according to group were depicted as bar graph. (B) Western blot for COX-2, 15-PGDH, iNOS, and p-NF- κ B p65 according to group. Mean expressions levels after triplicate measurement according to group. (C) Western blot for p-AKT, p-STAT3, and SOCS-3 according to group. Mean expressions levels after triplicate measurement according to group.

has been known as enzyme disposing tumorigenic PGE₂, thus playing tumor suppressor action in gastrointestinal (GI) system. These expressions of 15-PGDH were significantly preserved in 5.1 g/kg cpkimchi treated group ($p < 0.01$, Fig. 2B), while significantly decreased in Group 2. Notably, the levels of p-NF- κ B were all significantly decreased in group treated with kimchi (Fig. 2B). As noted in pathology according to each group, p-AKT were significantly increased in Group 2, but significantly decreased in cpkimchi treated group ($p < 0.01$, Fig. 2C), considering the contribution of STAT3 and SOCS-3, cpkimchi group showed significant ameliorating action of *H. pylori*-associated atrophic changes and ensuing tumorigenesis (Fig. 2C). Conclusively from 24 weeks observation, kimchi exerted significant anti-inflammatory and tumor suppressive action, but better in fermented kimchi compared to kimuchi. While these results supported the beneficiary actions of fermented kimchi against chronic *H. pylori* infection, we intended to pull out further mode of action.

Search for prominent genetic changes relevant to rejuvenating action of cpkimchi with RNAseq analysis. After transcriptome profiling reported in previous publication,⁽¹⁵⁾

in this study, we aimed to explore more mechanisms implicated in cancer prevention of fermented cpkimchi. In order to find target genes implicated in cancer prevention with cpkimchi under *H. pylori* infection, we repeated RNAseq analysis. As genes, which were elevated in *H. pylori* infection, but not elevated with cpkimchi, were explored, 126 genes were identified ($p < 0.01$, Supplemental Fig. 1A and B*). With classifying the categories of genes, up-regulated with *H. pylori* infection, but normalized or lower maintained with co-treatment of cpkimchi, ER stress gene, oxidative stress gene, tissue regeneration gene, angiogenesis gene, etc. were drawn. According to heatMap and gene ontology as shown in Supplemental Fig. 1A and B*, some genes implicated in endoplasmic reticulum stress (ER stress) were validated with RT-PCR (Supplemental Fig. 1C*), signifying the mitigating action of cpkimchi against ER stress. Next, as genes, which were down-regulated in *H. pylori* infection, but go-up with cpkimchi, total 262 genes were identified ($p < 0.01$, Supplemental Fig. 2A and B*), showing Heatmap). The categories of genes, down-regulated with *H. pylori* infection, but normalized or up-regulated with co treatment of cpkimchi were shown in Supplemental Fig. 2B*, containing cellular defense response

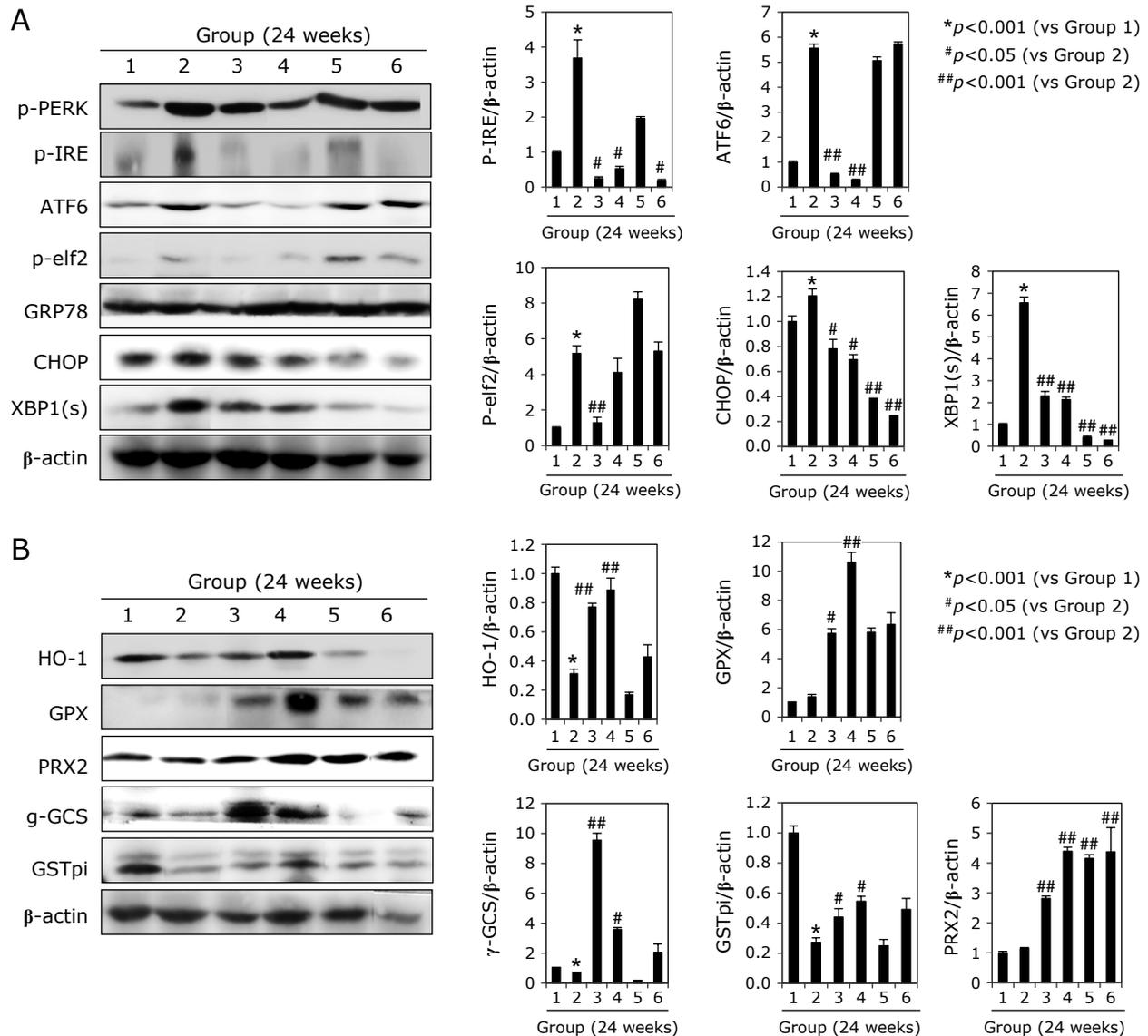


Fig. 3. The changes of ER stress and antioxidant genes explored at mucosal homogenates from 24 weeks model. (A) Western blot for ER stress protein according to group including pPERK, p-IRE, ATF6, p-elf2, GRP78, CHOP, and XBP. (B) Western blot for host defensive antioxidant proteins including GPX, PRX2, HO-1, γ -GCS, and GST.

gene, tissue regeneration gene, antioxidative gene, cell adhesion gene, etc. Among these, as seen in Supplemental Fig. 2C*, cpkimchi induced significant levels of heme oxygenase-1 (HO-1) and NAD(P)H quinone dehydrogenase 1 (NQO1) ($p < 0.001$).

Mitigation of ER stress and potentiation of antioxidative condition as additional rejuvenating mechanism of cpkimchi. Taken together the above outcomes of 24 weeks that cpkimchi led to significant rejuvenation of *H. pylori* initiated-, high salt promoted-CAG and prominent gene changes implicated in reliever of ER stress and antioxidative action via cpkimchi outlined by RNAseq analysis, we have curiosity whether transcriptome profiling can be verified in each group. As seen in Fig. 3A, significantly increases in the expressions of ER stress sensor, phosphorylated protein kinase RNA-like endoplasmic reticulum kinase (*p-PERK*), phosphorylated eukaryotic initiation factor-2 (*p-IRE*), ER stress transcription factor activating transcription factor 6 (*ATF6*), phosphorylated eukaryotic translation initiation factor 2 alpha (*p-elf2*), c/EBP homologous protein (*CHOP*), and x-box binding protein 1

(*XBP1*) were noted in Group 2, compatible with findings from RNAseq analysis (Supplemental Fig. 1*). These genes implicated in ER stress were significantly ameliorated in Group 3 and Group 4, but not or a little in Group 5 and Group 6, signifying that fermented cpkimchi led to rejuvenation of CAG through relieving *H. pylori*-induced ER stress, concluding that fermentation of kimchi and our special recipe may affect these achievements. On Fig. 3B, we also measured the genes engaged in antioxidative response including HO-1, glutathione peroxidase (*GPX*), peroxiredoxin 2 (*PRX2*), gamma-glutamylcysteine synthetase (γ -*GCS*), and glutathione *S*-transferase (*GST*)-pi. Commonly significant decreases in these antioxidative genes were noted in Group 2, whereas these were all consistently increased in Group 3 and Group 4.

Fermented cpkimchi prevented *H. pylori*-induced gastric tumorigenesis. Supported with our previous publication that cpkimchi efficiently prevented either *H. pylori*-associated gastric carcinogenesis,⁽¹²⁾ we extended the current study up to 36 weeks

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under the specific aim that three kinds of kimchi, cpkimchi, skimchi, and kimuchi were compared their efficacies against *H. pylori*-initiated, high salted diet-promoted gastric carcinogenesis models (36 weeks, Fig. 4A). As seen in Fig. 4B, Group III as 1.7 g/kg cpkimchi administered for 36 weeks and Group IV as 5.1 g/kg cpkimchi administered for 36 weeks showed significantly ameliorated either gross lesion score ($p < 0.05$, Fig. 4B) or pathological scores ($p < 0.01$, Fig. 4C). our model of *H. pylori* initiated-, salt promoted-model led to prominent tumorigenesis based on atrophic gastritis, tumorigenesis more prevalent in junctional area of forestomach and glandular area, while significant ameliorated lesions were noted in Group III and Group IV, but not in Group V and Group VI, signifying fermented cpkimchi significantly prevented *H. pylori*-associated gastric carcinogenesis, but fermented skimchi or kimuchi did not at all. As in 24 weeks, we have measured the expression of *iNOS*, *IL-1 β* , *RANTES*, *VCAM*, and *ICAM* mRNA according to group and found fermented cpkimchi, Group III and Group IV, led to significantly decreased expressions of these mediators ($p < 0.05$, Fig. 5A). Representational signals including ERK1/2 were significantly increased in Group II, but significantly inactivated in Group III and Group IV ($p < 0.05$, Fig. 5B). 15-PDGH as tumor suppressive protein was significantly preserved in Group III and Group IV accompanied with significantly decreases of p-AKT and β -catenin (Fig. 5C).

Additional chemopreventive mechanism of fermented cpkimchi; abrogated ER stress and apoptotic condition. Also, stimulated with the above results that cpkimchi led to significant amelioration of *H. pylori*-initiated, high salt-promoted gastric tumorigenesis and prominent gene changes implicated in ER stress and apoptosis action via cpkimchi figured by RNAseq analysis, we measured these gene changes in each group. As seen in Fig. 6A, significantly increases in the expressions of p-PERK, ATF6, CHOP, and XBP1 were noted in Group II (36 weeks), compatible with findings from RNAseq and these genes implicated in ER stress were significantly ameliorated in Group III and Group IV, but not or a little in Group V and Group VI, signifying that relieving ER stress with cpkimchi, fermented, exerted significant prevention of *H. pylori*-associated gastric tumorigenesis, considering the differences between Group III/IV and Group VI, fermentation is significantly implicated in cancer preventive action of kimchi. The other genes implicated in apoptosis contributed to cancer prevention of fermented cpkimchi as reflected with findings shown in Fig. 6B that significant increases in PARP cleavage, caspase-3 cleavage, decreases in BCL2 contributed to cancer prevention of cpkimchi.

Discussion

In the current study, we found, for the first time, that long-term dietary intake of kimchi can either rejuvenate *H. pylori*-associated CAG or prevent gastric carcinogenesis. However, though dietary intake of kimchi can contribute to amelioration of *H. pylori*-associated gastric diseases, they should be fermented to yield enough probiotics and should be enriched with cancer preventive gradients. Surprisingly, kimuchi rather aggravated *H. pylori*-associated gastric disease, signifying that probiotic and profuse phytochemicals-enriched recipe kimchi only can be beneficial against *H. pylori* infection (Fig. 7). Furthermore, in the current study, we could add the plausible mechanisms how cpkimchi can fight against *H. pylori* infection that either relieving ER stress and mitigating oxidative stress or imposing apoptotic mechanisms as core rejuvenating and cancer preventive actions of fermented cpkimchi in chronic *H. pylori* infection.

We performed RNAseq analysis to discover the transcriptomes implicated in cancer preventive actions of cpkimchi under the *H. pylori* infection (Supplemental Fig. 1 and 2*). Though still caveats exist, RNAseq is heralding a new period in

transcriptomics and is bringing much needed sensitivity and discrimination to global gene expressions assays.⁽¹²⁾ RNAseq analysis ranges from classic evaluations of differential transcript expression between samples to more diverse implications like gene expression dynamics, gene boundaries, and translation efficiency.⁽¹³⁾ For instance, Cheng *et al.*,⁽¹⁴⁾ using RNAseq analysis, identified the potential genes involved in tumosphere formation of colorectal cancer (CRC), which was EGF-STAT3 signaling pathway as stemness genes among EGFR positive CRCs, which can be a putative therapeutic target for colorectal cancer. As other example to clarify the usefulness of RNAseq, since microglia undergo dramatic changes to their expression patterns with regard to mitochondrial activity, Bosco *et al.*,⁽¹⁶⁾ revealed that microglia initiate immunological activity such as increasing interferon beta responsiveness to kainic acid-induced seizures. Conclusively, it is generally documented that the dominance of RNAseq over microarray in gene expression research has been acknowledged by multiple studies.⁽¹⁷⁾ In the current study, as seen in Supplemental Figures, statistically significant transcriptomes were identified, that is, total 943 genes were filtered to be different between before and after *H. pylori* infection, among which gene categories were presented in Supplemental Fig. 1A* and 68 genes were filtered different between *H. pylori* infection and *H. pylori* infection under cpkimchi (Supplemental Fig. 1B*). Further detailed categories were summarized and validated in Fig. 3 and 6. There was significant correlation between Heatmap figure and detailed RT-PCR or Western blot of each gene.

According the analysis of animal model irrespective of gastric pathology at different time point combined with validating separate RNAseq in the current study, significant implication of ER stress related genes was noted after *H. pylori* infection. Generally, since ER is an organelle for protein synthesis, folding and modification, lipid synthesis and calcium storage,⁽¹⁸⁾ ER under the stressful condition like chronic *H. pylori* infection leads to the accumulation of mis- or un-folded-proteins in the ER lumen, so called unfolded protein response (UPR) occurred, in order to restores ER homeostasis. Therefore, the UPR is characterized by three distinct downstream signaling pathways that promote cell survival or apoptosis depending on the stressor, and duration of ER stress. On detailed processing of ER stress, ER stress perturbations have been associated with excessive protein synthesis, abnormal ER calcium content, lipid overload, hypoxia, oxidative stress, iron imbalance, nutrient deprivation, cancer, and infection.⁽¹⁹⁾ ER stress in mammalian cells is recognized by the ER-resident proteins inositol requiring enzyme 1 (IRE1), protein kinase R (PKR)-like ER kinase (PERK), and ATF6, controlling transcriptional and translational responses to ER stress as central type-I transmembrane proteins of the UPR.⁽²⁰⁾ Since increased expression of pro-inflammatory cytokines during ER stress suggests roles of the UPR in inflammatory disorders and oxidative stress,^(21,22) after which *H. pylori* infection is one of cases imposing ER stress to stomach pathology.⁽²³⁾ Since *H. pylori* VacA could induce autophagy and increase cell death in human gastric cell lines, in which dilated ER accompanied with ER stress UPRs were observed in *H. pylori*-positive gastric mucosa. Reversely, knock out of CHOP by siRNA resulted in inhibition of VacA-induced apoptosis. Also, silencing of the PERK gene attenuated VacA-mediated phosphorylation of eIF2- α , CHOP induction, expression of BH3-only protein *Bim* and *Bax* activation.⁽²⁴⁾ As much as induction of the UPR in the milieu of *H. pylori*-induced chronic inflammation, ER stress is known to associate with mucous metaplasia, a precancerous lesion stepping into *H. pylori*-induced cancer.⁽²⁵⁾ In our study, RNAseq analysis consistently showed significant implication of ER stress genes in *H. pylori*-induced gastric pathologies and these findings were clearly demonstrated in animal model. In this condition,

*See online. <https://doi.org/10.3164/jcfn.20-180>

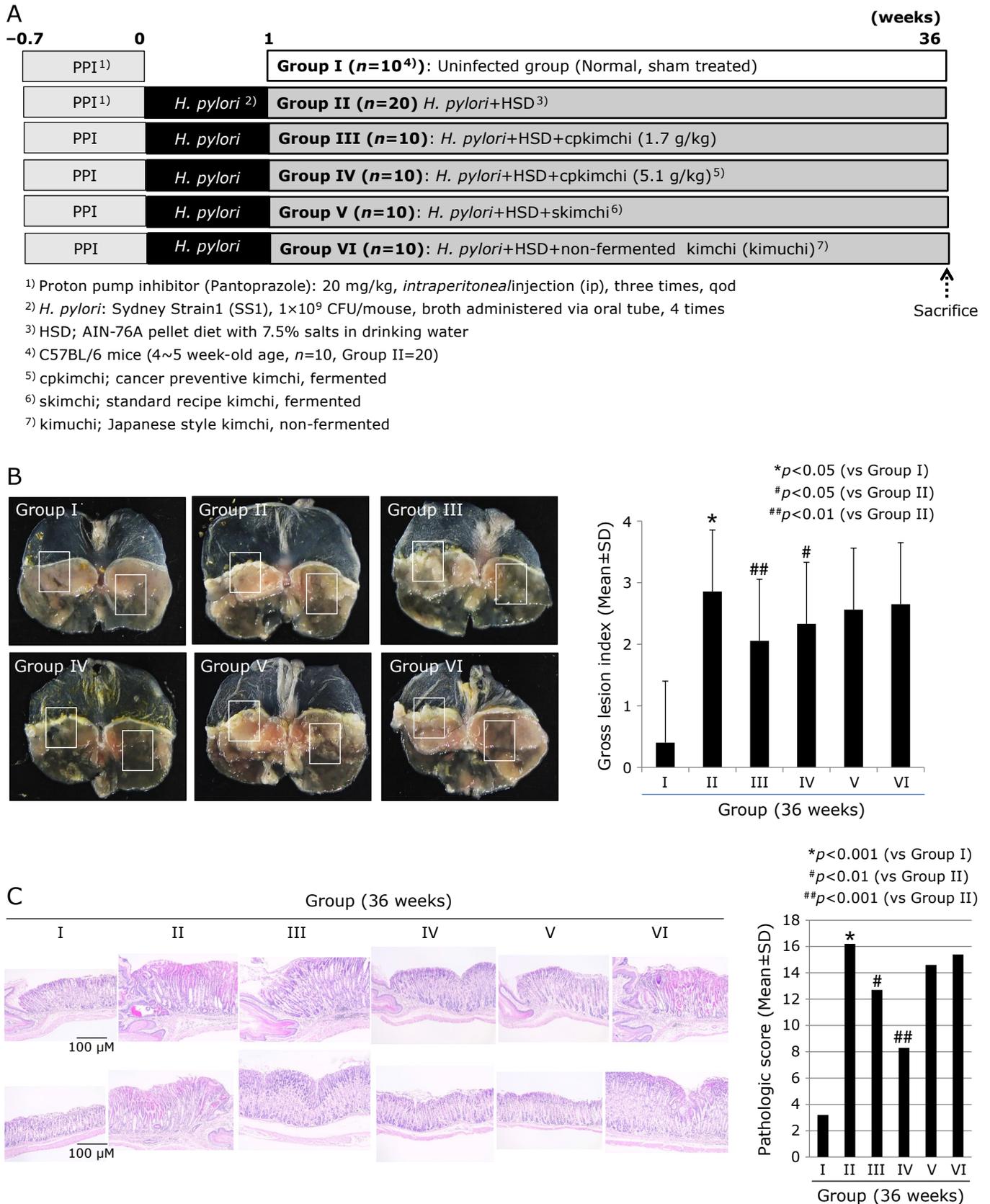


Fig. 4. Dietary intake of various kinds of kimchi on *H. pylori*-initiated, high salt diet-promoted gastric tumorigenesis, 36 weeks model. (A) Scheme for group, Group I as normal control, Group II as *H. pylori*-associated CAG control, Group III as disease control treated with 1.7 g/kg cpkimchi, Group IV as disease control treated with 5.1 g/kg cpkimchi, Group V as disease control treated with 5.1 g/kg skimchi, and Group VI as disease control treated with 5.1 g/kg kimuchi all sacrificed after 36 weeks of *H. pylori* infection. (B) Representative photo of resected stomach according to group and mean gross lesion scores according to group. (C) Representational pathology and mean pathological scores according to group, ×40 magnification. See color figure in the on-line version.

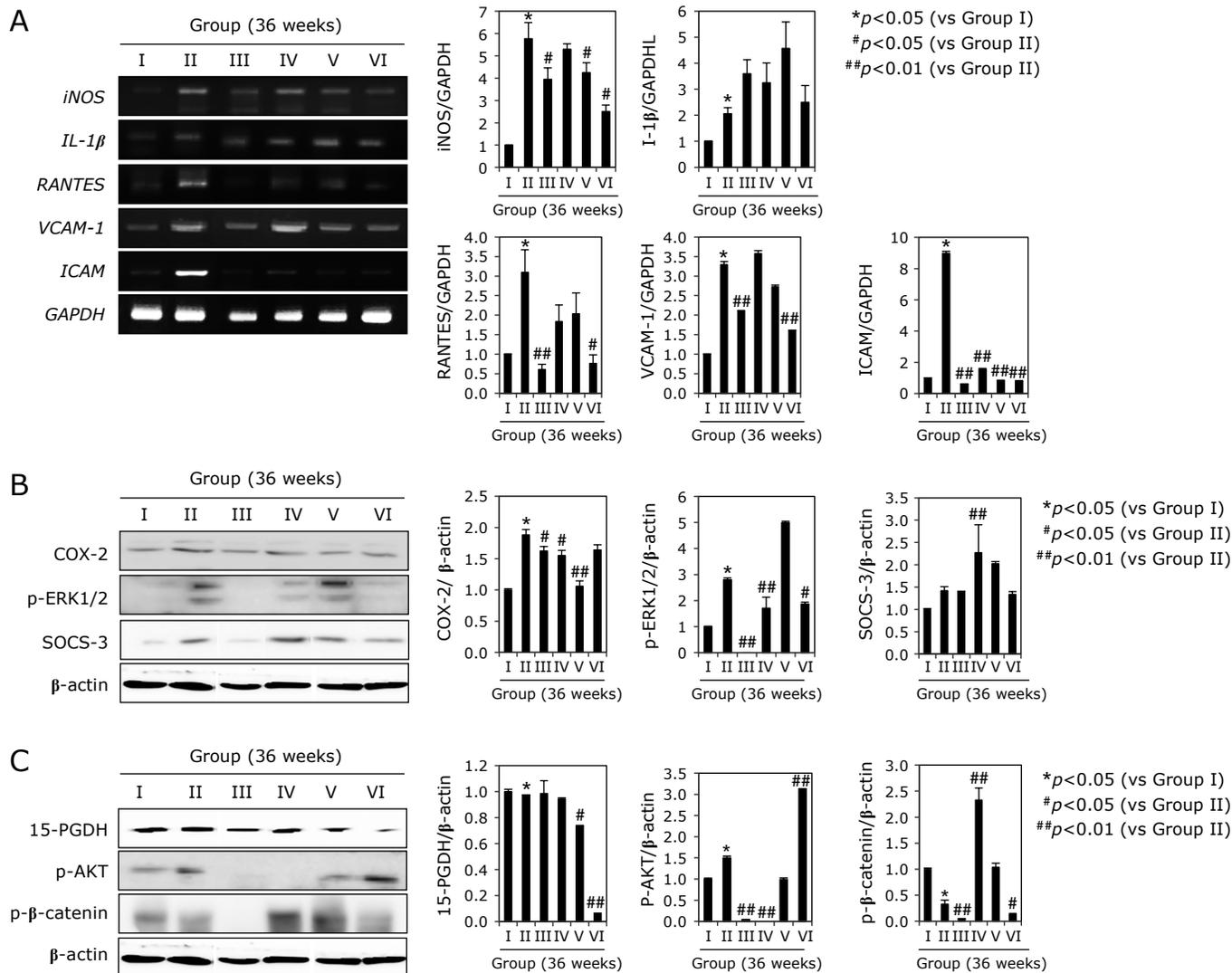


Fig. 5. Changes of inflammatory genes, p-ERK, SOCS-3, 15-PGDH, p-AKT, and p-β-catenin according to group (36 weeks). (A) RT-PCR for *iNOS*, *IL-1β*, *RANTES*, *VCAM-1*, and *ICAM* mRNA according to group. Mean expressions levels after triplicate measurement according to group. (B) Western blot for COX-2, p-ERK1/2, and SOCS-3 according to group. Mean expressions levels after triplicate measurement according to group. (C) Western blot for 15-PGDH, p-AKT, and p-β-catenin according to group. Mean expressions levels after triplicate measurement according to group.

cpkimchi significantly ameliorated either *H. pylori*-associated CAG or gastric tumorigenesis relevant to amelioration of ER stress genes. Though similar kimchi kinds, skimchi, and kimuchi were weak in these ameliorations accordant with lesser relieving of ER stress.

Next to ER stress amelioration relevant to cancer preventive effect of cpkimchi, RNAseq analysis significantly showed the intervention of oxidative stress in *H. pylori* infection and mitigation of oxidative stress based on antioxidative action of cpkimchi. Oxidative stress relevant to pathogenesis of *H. pylori* infection and associated gastric carcinogenesis had been proved in many publications, by which antioxidants like vitamin C, vitamin E, and rebamipide had been tried for either enhancing eradication rate or relieving gastric inflammation in animals and human trial. Kimchi also had been stressed to be antioxidative effects.⁽²⁶⁻²⁹⁾ Oxidative stress in *H. pylori* infection is defined as an imbalance between excessive production of reactive oxygen and nitrogen species and depletion of antioxidative system to eliminate the reactive intermediates.⁽³⁰⁾ As shown in Fig 4, cpkimchi significantly enriched antioxidative defense system

against chronic *H. pylori* infection, especially at 24 weeks of chronic *H. pylori* infection.

In accordant with antioxidative and ameliorating action of ER stress, significant anti-tumorigenic action of cpkimchi was attributed to apoptotic action (Fig. 7). At the 36 weeks of dietary administration of cpkimchi, significant induction of apoptosis led to significant amelioration of *H. pylori*-induced gastric tumorigenesis. Already we have documented that ER stress and consequent autophagy is involved in the cell death induced by *H. pylori*.⁽²³⁾ However, these apoptotic response is responsible for erosive, ulcerative, and ulcer recurring action relevant to *H. pylori* infection in earlier stage of infection. Also atrophic gastritis is caused by apoptosis of parietal cells or possible regenerating stem cells. On the other hand, the failure of apoptotic cell death under chronic mutagenic inflammation provides the foci of proliferation and tumorigenesis. Though revealed in part, still we have a curiosity how they modulate host immunity, persist life-long infection, and contribute to tumorigenesis and a little known how they are associated with extra-gastric diseases.⁽³¹⁾ Therefore, selective apoptosis of

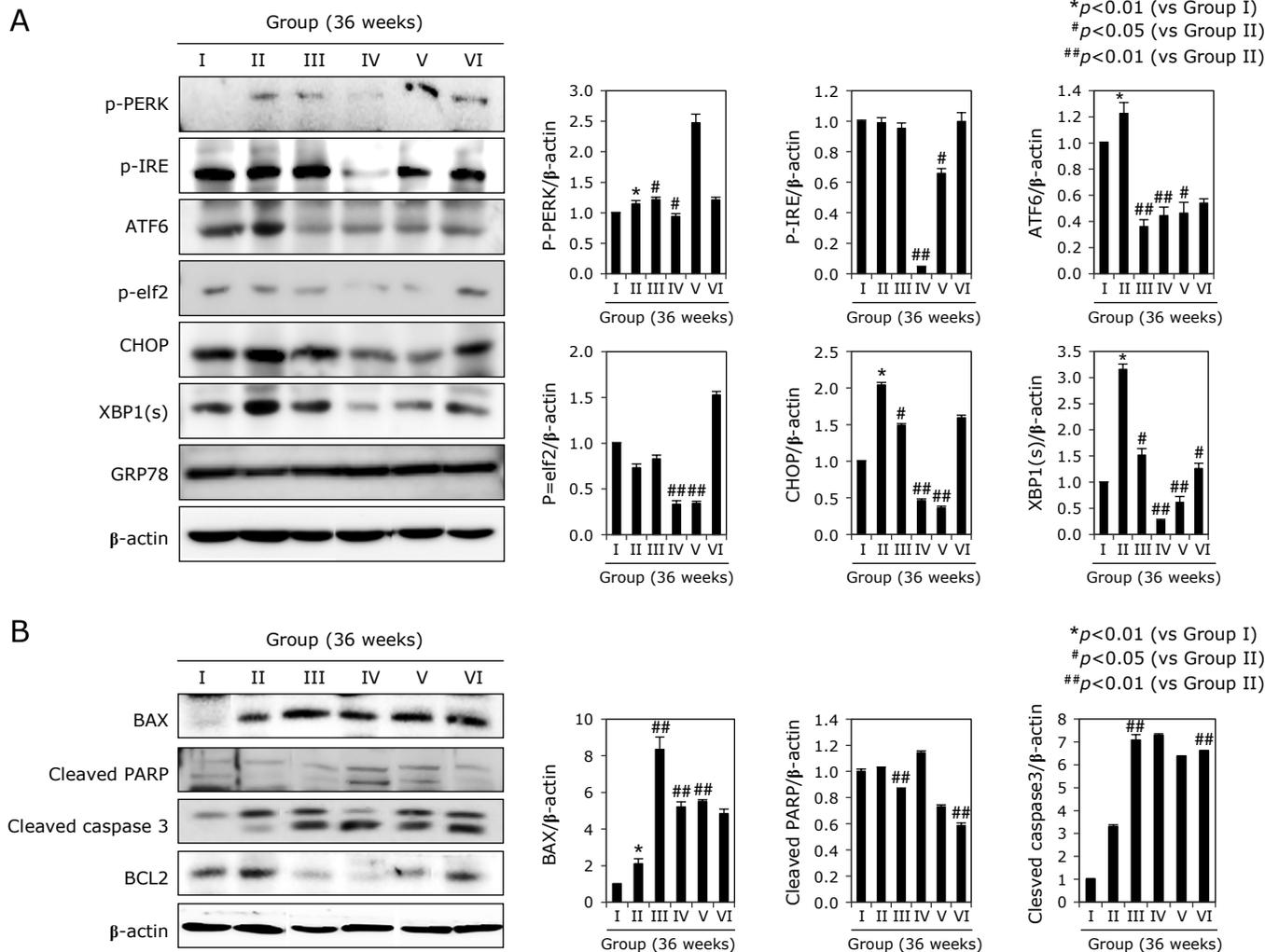


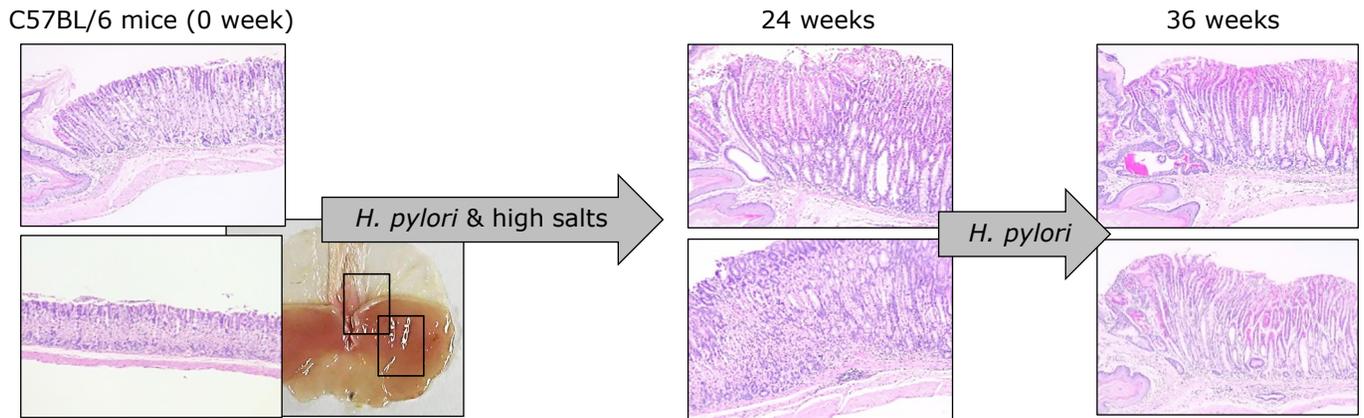
Fig. 6. The changes of ER stress genes and apoptotic genes explored at mucosal homogenates from 36 weeks model. (A) Western blot for ER stress protein according to group, p-PERK, p-IRE, ATF6, p-elf2, CHOP, XBP1, GRP78. (B) Western blot for apoptotic executors, BAX, cleaved PARP, cleaved caspase 3, BCL2.

proliferative and tumorigenic clone as well as anti-mutagenesis might be essential in preventing abnormal proliferation because all basis of resistance to apoptosis, immune evasion, and loss of cell junctions seen in *H. pylori*-infected host cells is essential in gastric carcinogenesis and regulation of apoptosis is remarkably impaired in atrophic gastritis associated with gastric cancer after *H. pylori* infection.^(32,33) According to our model, cpkimchi was excellent in these modulations of apoptosis to prevent tumorigenesis. As much as the implication of apoptosis as cancer preventive mechanisms, in the current study, we were very lucky to find the significant preservation of cancer suppressive enzyme relevant to COX, 15-PGDH with cpkimchi (Fig. 2B and 4B), more significant contribution in 24 weeks of *H. pylori* infection.

Lastly, probiotic kimchi contributed to cancer preventive as well as rejuvenating action of kimchi because kimchi did not affect at all in this matter. Though skimchi is a traditional Korean fermented side-dish containing rather diverse strains of lactic acid bacilli such as *Lactobacillus plantarum*, *L. acidophilus*, *L. curvatus*, *L. brevis*, *L. sakei*, *Leuconostoc mesenteroides*, *Enterococcus faecium*, and *Weissella cibaria* in addition to several prebiotics and the presence of vitamins, minerals, and dietary fibers,⁽³⁴⁻⁴¹⁾ our study might be the first document to prove the probiotic food contributed to

beneficial outcome in preventing precancerous and cancer in the stomach. Although specific probiotics are beneficial in some GI problems, but still many of the new publications did not report clear benefits of probiotics. For instance, some probiotics can relieve GI symptoms in irritable bowel syndrome, prevent antibiotics-associated diarrhea, and *H. pylori* eradication therapy, but some are below real significance.⁽⁴²⁻⁴⁴⁾ Though still remains room for further clarification, it is generally agreed that probiotics as an adjuvant treatment in *H. pylori* eradication therapy is generally agreed,⁽⁴⁵⁻⁴⁷⁾ but our study clearly showed the presence of probiotics via fermentation might play key role in cancer prevention.

As the limitation of the current study, the current result is proof of concept study necessitating the well-designed clinical trial whether kimchi administration really rejuvenates atrophic gastritis or prevents the progression of precancerous gastric lesion. In this journal of clinical biochemistry and nutrition, we publish data showing the significant microbiome changes compatible with current *in vivo* animal study after 10 weeks of cpkimchi administration. Though not studied in this experiment, significant changes in fecal microbiota were noted with kimchi administration., further stressing the importance of fermentation to yield probiotics. In addition to anti-inflammatory and anti-



+fermented kimchi intake rejuvenated chronic atrophic gastritis (CAG)

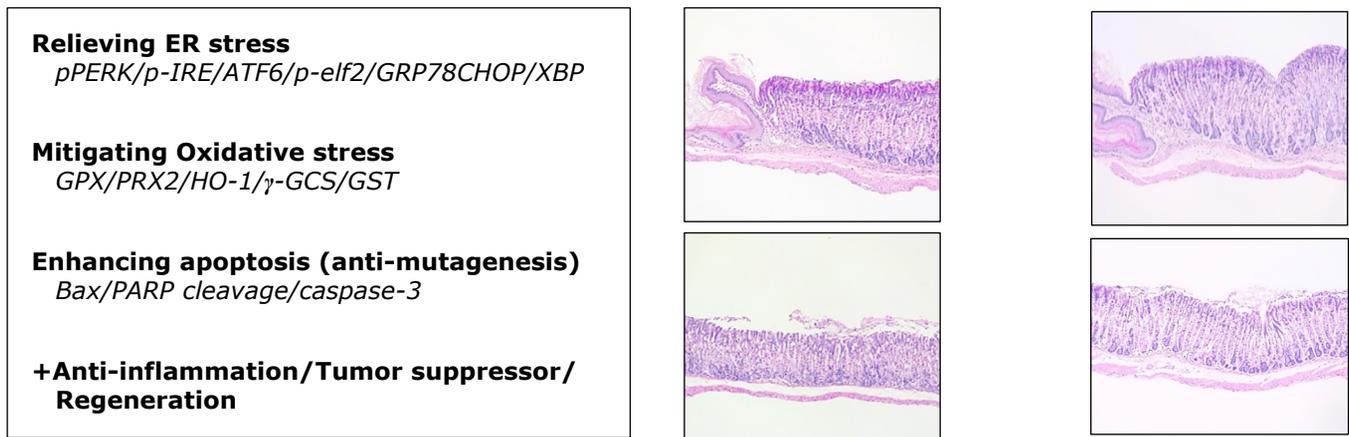


Fig. 7. Schematic summary. Against *H. pylori* initiated, salts-promoted CAG and gastric tumors, intake of kimchi as dietary manner significantly rejuvenated CAG (24 weeks) and prevented gastric tumorigenesis (36 weeks). Using gastric homogenates obtained from either 24 weeks or 36 weeks, the changes of inflammatory genes, tumor suppressor genes, and oxidative stress related genes were measured, after whichi significant anti-inflammatory, anti-mutagenic, and significant regenerative actions were operated by dietary intake of kimchi. Furthermore, RNAseq analysis suggested novel mechanisms of ER stress, oxidative stress, and irregulated cell proliferation were implicated in *H. pylori* infection. In this exploration, kimchi led to significant ameliorating action of ER stress, mitigating action of oxidative stress, and regenerating mechanisms. Though not touched in the current experiment, fermentation seems to be critical in these beneficiary action of kimchi against *H. pylori* infection. See color figure in the on-line version.

mutagenic action of kimchi, relieving ER and oxidative stress relevant to *H. pylori* infection was documented for the first time, but detailed evidences using mouser model of ER or oxidative stress should be followed. Also, if we extended the current study up to 45–50 weeks, we could present cancer preventive efficacy of kimchi, we sacrificed mice at 36 weeks.

Dietary intake of cancer preventive phytochemicals or nutrients seems to be the ideal way in three points of benefits, the first as safety and no side effect, the second as high compliance, and the last physiologic contribution. So many trials in this matter had been tried with some efficacy, but the features of our trial are well-documented proof of concept, translational achievement.⁽⁴⁸⁾ Our and other investigators believe that the diet may play a critical role in defining the final outcome of *H. pylori* infection particularly if certain intake of dietary components is continued for a long time. However, despite a recent surge in research related to the role of dietary ingredients, well-designed, large-scale clinical trials are required to give clinical benefits. Therefore, since the final conclusion can be reached after the successful documentation of randomized clinical trial, randomized clinical trial (RCT) is under progress in our department to document placebo-controlled, double-blinded

clinical trial to measure the efficacy of well-fermented, specially recipe cpkimchi in patients with *H. pylori*-associated dyspepsia with changes of fecal microbiota, functional dyspepsia-quality of life, and assessment of gastritis. Before this, we conclude dietary intake of cpkimchi can be anticipated strategy either to rejuvenate *H. pylori*-associated CAG or to prevent gastric cancer.

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Abbreviations

ATF6	ER stress transcription factor activating transcription factor 6
CAG	chronic atrophic gastritis

CHOP	c/EBP homologous protein
cpkimchi	cancer preventive kimchi
DEG	differentially expressed genes
ER stress	endoplasmic reticulum stress
GI	gastrointestinal
HO-1	heme oxygenase-1
MOI	multiplicity of infection
NQO1	NAD(P)H quinone dehydrogenase 1
15-PDGH	15-hydroxyprostanin dehydrogenase
p-elf2	phosphorylated eukaryotic translation initiation factor 2
p-IRE	phosphorylated eukaryotic initiation factor-2

p-PERK	phosphorylated protein kinase RNA-like endoplasmic reticulum kinase
RCT	randomized clinical trial
RNaseq	RNA sequencing analysis
skimchi	standard recipe kimchi
UPR	unfolded protein response
XBP1	x-box binding protein 1

Conflict of Interest

No potential conflicts of interest were disclosed.

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