

Transcriptome profiling analysis of the response to walnut polyphenol extract in *Helicobacter pylori*-infected cells

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Dietary intervention to prevent *Helicobacter pylori* (*H. pylori*)-associated gastric diseases seems to be ideal with no risk of bacterial resistance, safe long-term intervention, and correcting pathogenic mechanisms including rejuvenation of precancerous atrophic gastritis and anti-mutagenesis. A transcriptome as set of all RNAs transcribed by certain tissues or cells demonstrates gene functions and reveals the molecular mechanism of specific biological processes against diseases. Here, we have performed RNAseq and bioinformatic analysis to explain proof of concept that walnut intake can rescue from *H. pylori* infection and explore unidentified mode of actions of walnut polyphenol extract (WPE). As results, *BIRC3*, *SLC25A4*, *f3* transcription, *VEGFA*, *AZU1*, *HMOX1*, *RAB3A*, *RELB*, *TNIP1*, *ETFB*, *INPP5J*, *PPME1*, *RHOB*, *TPI1*, *FOSL1*, *JUND*, *RELB*, *KLF2*, *MUC1*, *NDRG1*, *ALDOA*, *ENO1*, *PFKP*, *GPI*, *GDF15*, and *NRTN* genes were newly discovered to be enriched with WPE, whereas *CCR4*, *BLNK*, *CCR7*, *CXCR4*, *CDO1*, *KLSG1*, *SELE*, *RASGRP2*, *PIK3R3*, *TSPAN32*, *HOXC-AS3*, *HCG8*, *BTNL8*, and *CXCL3* genes as inhibitory targets by WPE in *H. pylori* infection. We identified additional genes what WPE afforded actions of avoiding *H. pylori*-driven onco-inflammation and rejuvenating precancerous atrophic gastritis. Conclusively, after applying RNAseq analysis in order to document walnut intake for precision medicine against *H. pylori* infection, significant transcriptomic profiling applicable for validation were drawn.

Key Words: *H. pylori*, RNAseq, transcriptome, pharmanutrient, walnut polyphenol extract

The health promoting effects of walnuts plentiful of *n*-3 polyunsaturated fatty acids (*n*-3 PUFAs), fibers, and plentiful vitamins had been attributed to its anti-inflammatory, regenerative, and anti-oxidative properties against various clinical diseases such as metabolic, neurological, inflammatory, and degenerative diseases. Since we have published *Fat-1* transgenic mice over-expressing *3-desaturase* significantly mitigated various gastrointestinal cancer models including *Helicobacter pylori* (*H. pylori*)-associated gastric cancer, colitis-associated cancer, and hepatobiliary tract cancers based on anti-cancer and anti-mutagenic actions in both *in vitro* cell models as well as *in vivo* animal models benefited through *n*-3 PUFAs synthesized within cells/tissues.⁽¹⁻⁵⁾ As representational mechanisms of walnut containing high levels of *n*-3 PUFAs, same as seen in *Fat-1* TG mice, significant inhibitory actions of inflammatory genes such as COX-2/PGE₂/NF-κB/IL-6/STAT3, whereas significant inducing actions of defensive proteins such as HO-1/Nrf2/PPAR-γ/SOCS-1/15-PGDH were noted after walnut administration in *H. pylori* infection to mitigate gastric

damages.^(3,4,18)

Application of next generation sequencing (NGS) technologies exploiting whole genome sequencing to targeted sequencing has played an important role in precision medicine via the identification of the genetic variations and anomalies in a high throughput analysis (HTP), cost effective, faster technology, and a more comprehensive and accurate tool for genome analysis, simply “go-to-technology”,^(6,7) in which all the four NGS-based approaches, i.e., whole genome sequencing,⁽⁸⁾ whole exome sequencing,⁽⁹⁾ RNA sequencing (RNAseq),^(10,11) and targeted sequencing⁽¹²⁾ have been exploited for the detection of the genetic and epigenetic changes implicated in gastric carcinogenesis, among which RNAseq analysis is known to be more sensitive than microarray platform, very robust, precise, and highly quantitative, by which RNAseq analysis is being used to study the dynamics and complexity of eukaryotic transcriptomes, giving new biological insights into the active genome, fulfilling “seeing is believing”, applied for transcriptome profiling challenged with nutrient or discovered as biomarker.^(13,14) Furthermore, with recent revolutionized advance of transcriptome technology and bioinformatic analysis, single-cell RNAseq technology allows the dissection of gene expression at single-cell resolution, including quality control, read mapping, gene expression quantification, batch effect correction, normalization, imputation, dimensionality reduction, feature selection, cell clustering, trajectory inference, differential expression calling, alternative splicing, allelic expression, and gene regulatory network reconstruction.⁽¹⁵⁻¹⁷⁾

All together with achievements of our research group documenting that dietary intake of walnut or treated with walnut polyphenol extract (WPE) can significantly prevented either *H. pylori*-associated precancerous chronic atrophic gastritis (CAG) or developments of gastric tumorigenesis including gastric cancer as chemopreventive way,⁽¹⁸⁾ in parallel with beneficiary actions of *n*-3 PUFAs drawn from *Fat-1* transgenic mice synthesizing *n*-3 PUFAs, in this study, we performed RNAseq analysis for pulling-out transcriptome profiling in order to fulfill precision medicine against troublesome *H. pylori* infection by dietary walnut intake and found that WPE significantly can achieve either amelioration of “Prof. Correa P’s great hypothesis”⁽¹⁹⁻²²⁾ that *H. pylori* led to “chronic superficial gastritis-CAG-intestinal metaplasia-gastric adenoma-gastric adenocarcinoma” or the Japanese government effort that “the eradication strategy is a way for prevention”. Successfully, we explored gene profiling analyses to uncover

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regulatory relationship between gene and track the trajectories to document the beneficiary mechanisms of WPE against *H. pylori* infection and we could discover the contributing core genes to explain cancer preventive actions of walnut.

Materials and Methods

Materials. RPMI-1640 medium, fetal bovine serum, penicillin (FBS), streptomycin were products of Gibco BRL (Grand Island, NY) and materials for culturing *H. pylori* were sheep blood agar, GasPak™ and anaerobic jars (BD Biosciences, Sparks, MD).

***H. pylori* culture.** *H. pylori* strain ATCC 43504 (American Type Culture Collection, *cagA*⁺ and *vacA* s1-m1 type's strain) was used. *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 3–5 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 10⁹ colony-forming units (CFUs)/ml.

Preparation of walnut polyphenol extract. WPE from English walnuts (*J. regia*, California Walnut Commission) was prepared according to a previously described methanolic extraction method.⁽²³⁾ Briefly, after the walnuts were frozen for 24 h, the shelled kernels were finely ground and immersed in a solution of 75% acetone containing 526 μm/L sodium metabisulfite. The solution was subsequently purged with N₂ to prevent oxidation and was incubated at 4°C. After 24 h, the solution was decanted, thereby resulting in a cold extract that was centrifuged at 8,000 × *g* for 10 min. The resulting supernatant was filtered using Whatman filter paper No. 2. To remove lipids from the sample, the acetone was removed under reduced pressure and methanol (50% aqueous, vol/vol) was added. After three consecutive hexane extractions, the extracts were lyophilized to a dry powder after removing the methanol to prevent oxidation. All of the prepared sample were stored at 80°C in acidic condition until needed or prepared freshly on the day when WPE treatment is needed in order to avoid from either peroxidized or denatured extracts.

Bacteria strain and infection condition. *H. pylori* (ATCC 43504) with the typical S shape, gram-negative rods, possessing the *CagA* and *VacA* were provided in a frozen state by ATCC. *H. pylori* ATCC 43504 strains were grown on tryptic soy agar with 5% sheep blood agar (BD Diagnostics) and Dent antibiotics supplement (Oxoid) at 37°C under microaerophilic conditions (Campy-Pak 273 System, BBL). RGM1 cells were incubated overnight in fresh serum- and antibiotic-free RPMI 274 medium and were infected with *H. pylori* at multiplicities of infection (MOI) of 100:1 for 48 h. Though we found no influence of WPE on *H. pylori* culture, we repeated the status of *H. pylori* colonization in group treated with WPE in the presence of *H. pylori* and *H. pylori* infection alone before RNA isolation for RNAseq analysis.

Cell Culture. AGS cells were purchased from ATCC (Manassas, VA), where the cells were properly stored and routinely authenticated (including DNA fingerprinting). 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). All mediums supplemented with 10% fetal bovine serum (Gibco BRL) at 37°C in 5% CO₂. AGS cells were pretreated with WPE for 1 h and stimulated with 100 MOI *H. pylori* for 48 h. The control group was loaded with the same concentration of the dissolving media of DMSO.

RNA isolation. Total RNA was isolated using Trizol reagent (Invitrogen). RNA quality was assessed by Agilent 2100 bio-analyzer using the RNA 6000 Nano Chip (Agilent Technologies, Amstelveen, The Netherlands), and RNA quantification was performed using ND-2000 Spectrophotometer (Thermo Fisher Sci., Wilmington, DE).

RNAseq. For control and test RNAs, the construction of library was performed using QuantSeq 3'mRNA-Seq Library Prep Kit (Lexogen, Inc., Vienna, Austria) according to the manufacturer's instructions. In brief, each 500 ng total RNA were prepared and an oligo-dT primer containing an Illumina-compatible sequence at its 5' end was hybridized to the RNA and reverse transcription was performed. After degradation of the RNA template, second strand synthesis was initiated by a random primer containing an Illumina-compatible linker sequence at its 5' end. The double-stranded library was purified by using magnetic beads to remove all reaction components. The library was amplified to add the complete adapter sequences required for cluster generation. The finished library is purified from PCR components. High-throughput sequencing was performed as single-end 75 sequencing using NextSeq 500 (Illumina, Inc., San Diego, CA).

Data analysis and pathway analysis. QuantSeq 3'mRNA-Seq reads were aligned using Bowtie2.⁽²⁴⁾ Bowtie2 indices were either generated from genome assembly sequence or the representative transcript sequences for aligning to the genome and transcriptome. The alignment file was used for assembling transcripts, estimating their abundances and detecting differential expression of genes. Differentially expressed gene were determined based on counts from unique and multiple alignments using coverage in Bedtools.⁽²⁵⁾ The RC (Read Count) data were processed based on quantile normalization method using EdgeR within R (R development Core Team, 2016) using Bioconductor.⁽²⁶⁾ Gene classification was based on searches done by DAVID (<https://david.abcc.ncifcrf.gov/>) and Medline databases (<https://www.ncbi.nlm.nih.gov/>).⁽²⁷⁾ Pathway analysis was performed on differentially expressed genes based on the Kyoto Encyclopedia of Genes and Genome pathway (KEGG) databases.⁽²⁸⁾

Statistical analysis. The data are represented as mean ± SD of the experiments. Either Student's *t* test or a two-way analysis of variance with a post-hoc test was performed to determine the differences between the groups using a commercially available program (SPSS 12 for Windows; SPSS Inc., Chicago, IL). The level of significance was 0.05.

Results

RNAseq analysis to find genes participated in *H. pylori* infection and regulated by WPE in the presence of *H. pylori*. Before the current study, we have published WPE significantly inhibited STAT3 activation via regulating IL-6/IL-6R/JAK signaling, for which WPE significantly activated PPAR-γ/SOCS1 to inhibit STAT3.^(18,29–31) In previous *in vivo* animal model of *H. pylori* infection, we have successfully documented that dietary intake of walnut in pellet diet significantly decreased *H. pylori*-induced gastric inflammation and rejuvenation of *H. pylori*-associated CAG, concluding walnut seems to be of potential factor to regulate *H. pylori* infection.⁽¹⁸⁾ Supported with these previous outcomes, in this study, we performed RNAseq analysis to find genes in this beneficiary actions of walnut, genes increased with WPE in the presence of *H. pylori* and genes decreased with WPE in the presence of *H. pylori* infection and summarized in Fig. 10. As shown in hierarchical clustering analysis (heatmap) (Fig. 1B), 943 genes were filtered as significantly implicated in either change after *H. pylori* infection, but modulated with WPE in the presence of *H. pylori* infection. These genes were classified as shown in Fig. 1C according to up-regulated or down-regulated status. In this reaction, AGS cells were infected with *H. pylori* (100 MOI, 48 h) and very freshly extracted walnut polyphenols were applied. All reactions were repeated four times. 943 discovered genes were categorized according to gene function and status of gene expression, up and down and the analyses were all repeated in triplicate manner, of which analysis was repeated with pooling of samples as validation manner. Gene ontology

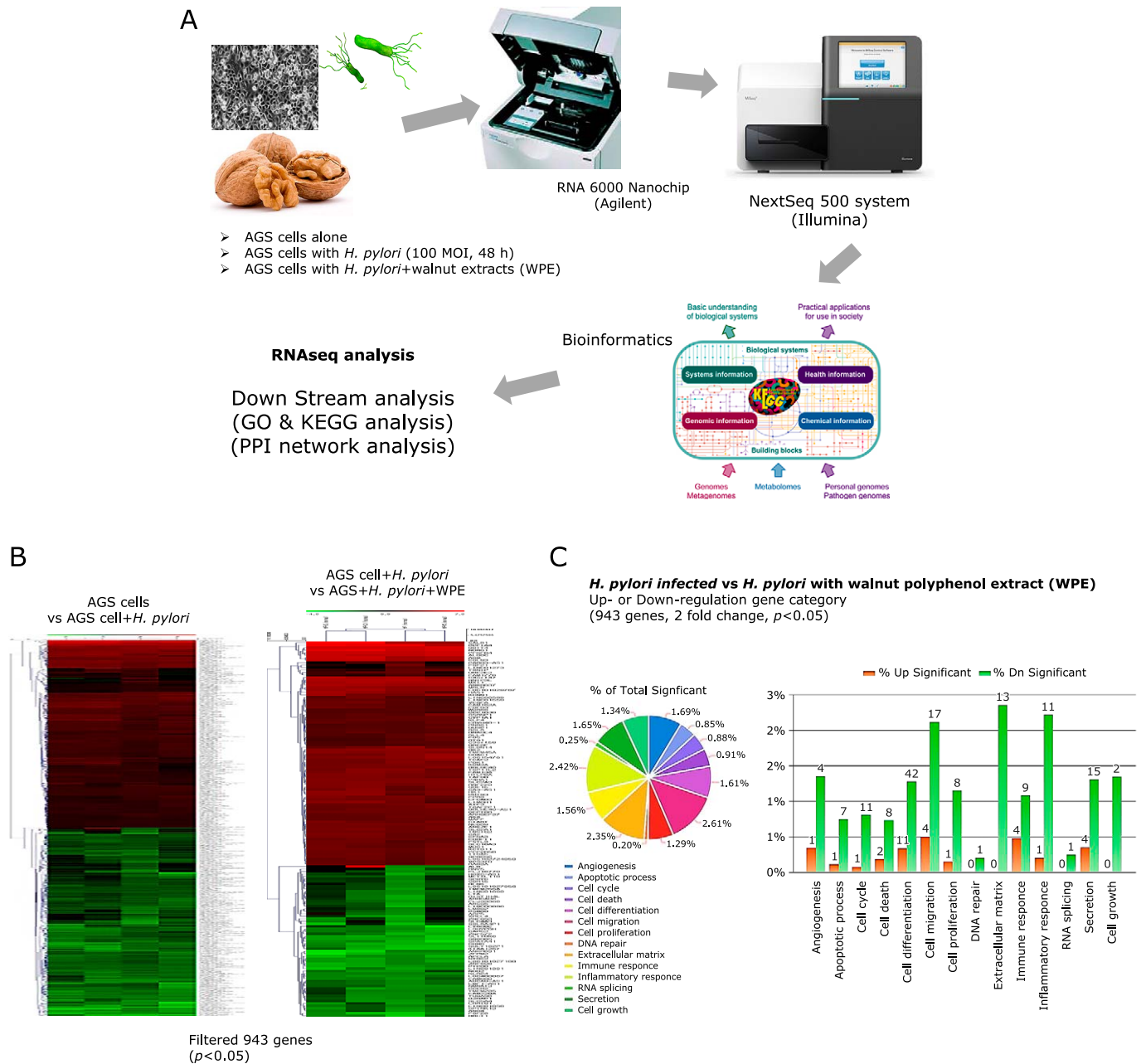


Fig. 1. RNAseq analysis in non-treated gastric cells, gastric cells infected with *H. pylori* infection, and cells infected with *H. pylori* under WPE (walnut polyphenol extracts) treatment (A) *in vitro* AGS cells infected with *H. pylori*, and infected with *H. pylori* in the presence of WPE, triplicate repeated, analyzed with NextSeq 500 (Illumina) after Agilent 6000 Nanochip for RNA qualification and quantification. (B) Results of hierarchical clustering analysis (heatmap) for differential expressed gene (DEG) analyzed according to significantly changed after *H. pylori* infection and infected with *H. pylori* in the presence of WPE, 943 genes included within $p < 0.05$. Red and green in the color bar represent high and low expression levels, respectively. Red and green in the y-axis represent upregulated and downregulated DEGs, respectively. (C) GO (gene ontology) function analysis of the upregulated/downregulated DEGs including biological process, cellular component and molecular function, gene analyzed according to significantly changed after *H. pylori* in the presence of WPE, 943 genes included showing $p < 0.05$. See color figure in the on-line version.

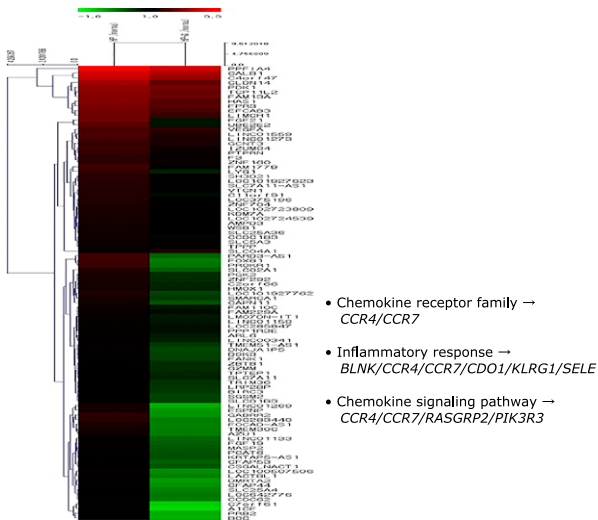
(GO) functional enrichment analysis and KEGG pathway enrichment analysis were performed on the DEGs (up- or downregulated) (Fig. 1C).

Genes category significantly different that elevated after *H. pylori* infection, but significantly decreased with WPE in the presence of *H. pylori* infection. Figure 2A showed heatmap comparing the genes of pooled samples featuring genes markedly decreased genes with WPE administration among genes significantly increased after *H. pylori* infection, denoting

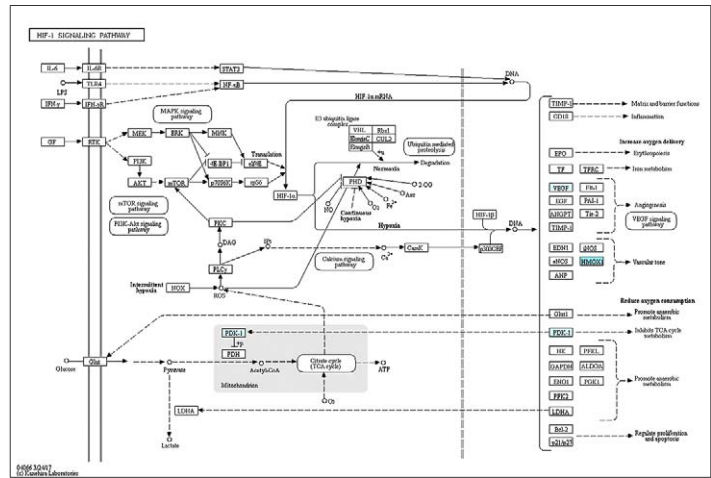
mitigating genes by walnut against damaging *H. pylori* infection, *C-C chemokine type 4 (CCR4)/CCR* as chemokine receptor family gene, *B cell linker protein (BLNK)/CCR7/CCR4/cysteine dioxygenase type 1 (CDO1)/killer cell lectin-like receptor subfamily G member 1 (KLRG1)/selectin E (SELE)* as inflammatory response, and *CCR4/CCR7/negative-feedback loop regulating ERK1/2 activation and mediation (RASGRP2)/phosphatidylinositol 3-kinase regulatory subunit gamma (PIK3R3)* as chemokine signaling pathway related genes. On KEGG analysis from heatmap, hypoxia-

A

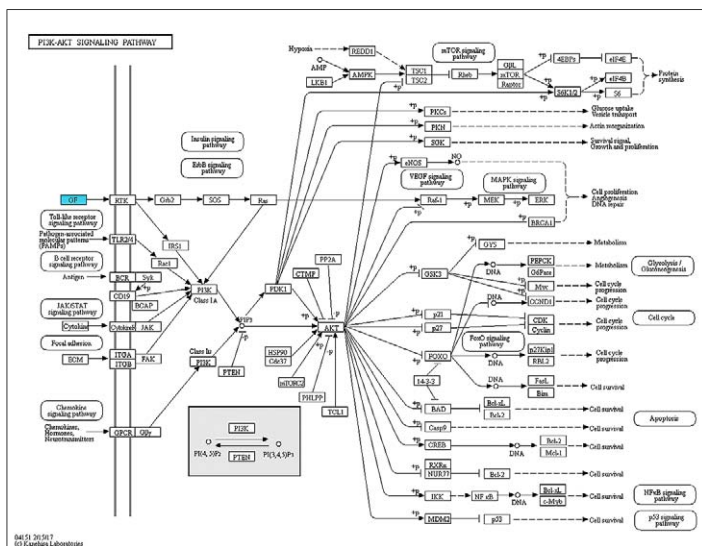
Genes category different significantly which showed ↑ *H. pylori* infection, but ↓ with WPE in the presence of *H. pylori* (filtered 91 genes, $p < 0.05$)



B



C



D

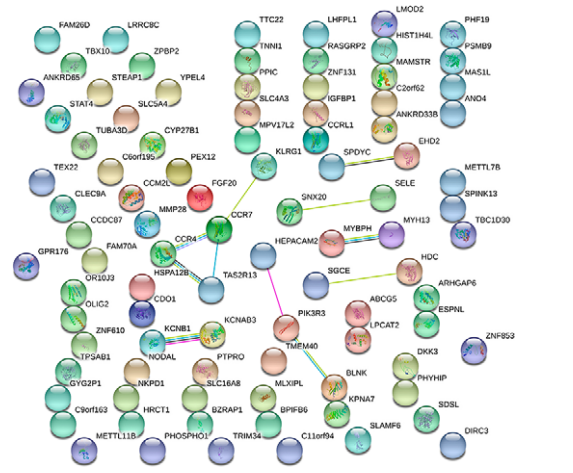


Fig. 2. (A) Expression heatmap for differential expressed genes showing significant trend like increased with *H. pylori* infection, but significantly decreased with co-treatment of WPE, description of gene category. GO enrichment analysis in box. (B,C) Kyoto Encyclopedia of Genes and Genomes enrichment analysis (KEGG) analysis. Annotation of KEGG HIF-1 α and PI3K signaling pathways with transcriptomic data. (D) STRING-generated interaction network of the differentially expressed genes Amplification of the network for PPI associated with CCR4. PPI network of DEGs shows red nodes represent DEGs up-regulated in the model group, while green nodes represent DEGs down-regulated in the model group. The color shade of a red node is positively correlated with the degree of this node, while the darker the color is, the higher the expression of up-regulated DEGs is. See color figure in the on-line version.

inducing factor (HIF) pathway (Fig. 2B) and PI3K pathway (Fig. 2C) highly linked with *H. pylori* infection and *H. pylori* infection in the presence of walnut extracts and inflammation-processing angiogenic activities on Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) Gene-Go analysis (Fig. 2D). On Fig. 3, we presented all gene status in bar analysis to compare AGS cells alone, AGS cells infected with *H. pylori*, and AGS cells infected with *H. pylori* in the presence of WPE. Conclusively, WPE significantly triggered genes to defense against *H. pylori*-infection-associated hypoxia, inflammation, apoptotic, and some mutagenesis.

Genes category significantly different that decreased after *H. pylori* infection, but significantly increased with WPE in the presence of *H. pylori* infection. Figure 4A showed heatmap comparing the genes of pooled samples featuring genes markedly increased genes with WPE among genes significantly decreased after *H. pylori* infection, denoting affording cytotective genes by walnut against *H. pylori* infection, briefly brief described, *Baculoviral IAP repeat containing 3* (BIRC3)/*solute carrier family 25 member* (SLC25A4) as regulatory genes of necroptotic process, *f3* transcription (F3)/*vascular endothelial growth factor A* (VEGFA)/*azurocidin 1* (AZU1) genes as positive

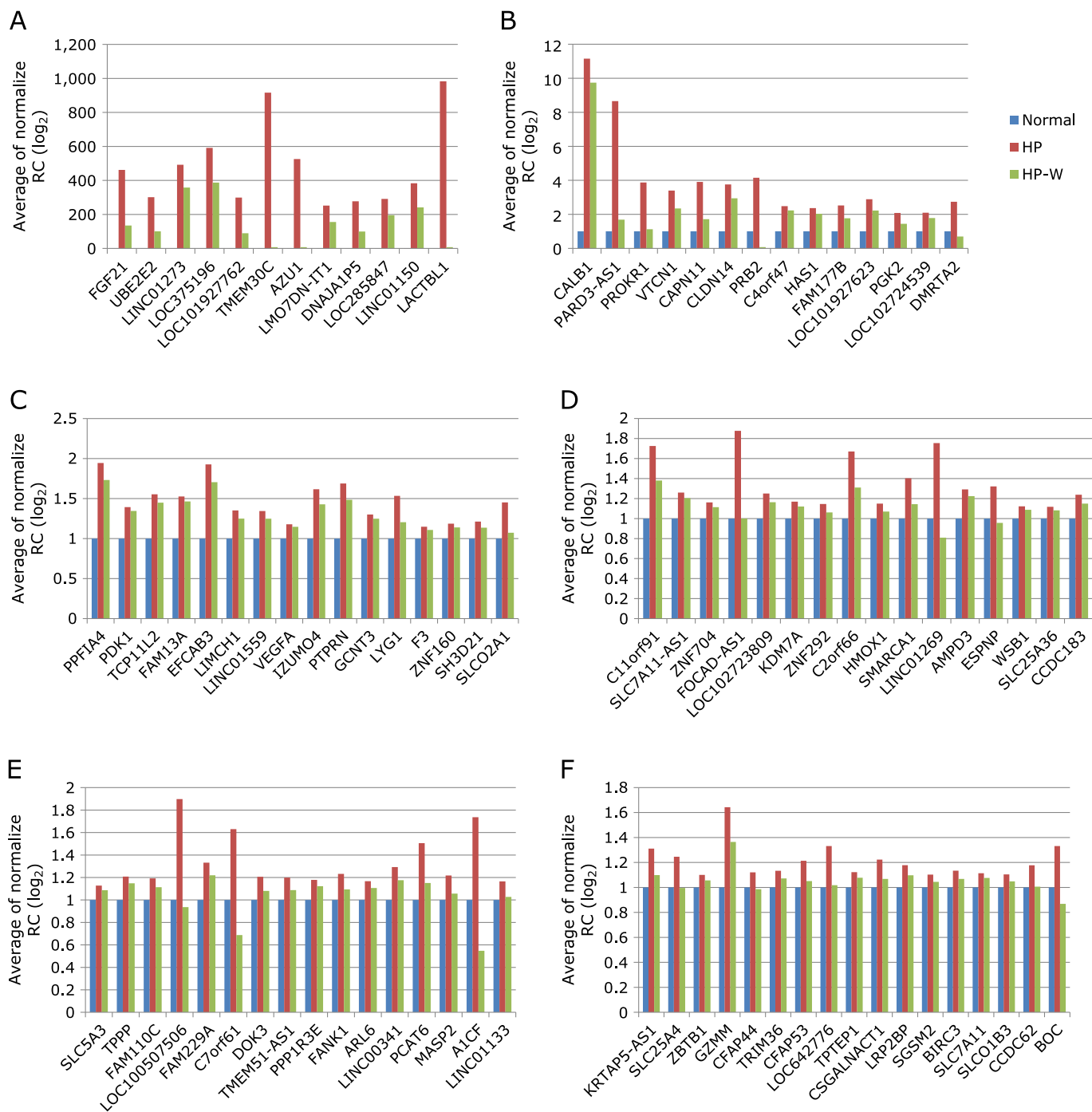


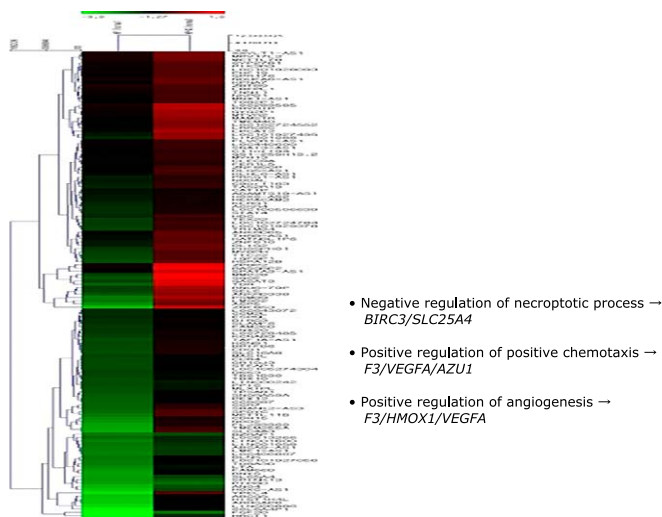
Fig. 3. Whole genes analyzed as significantly different, significantly increased after *H. pylori* infection, but significantly decreased after *H. pylori* infection in the presence of WPE ($p < 0.05$). HP, *H. pylori*; HP-W, *H. pylori* + WPE.

regulation of positive chemotaxis, and *heme oxygenase 1* (HMOX1)/VEGFA as positive regulation of angiogenesis. In this exploration, *H. pylori* retarded gene response in restoring angiogenesis and regeneration as well overwhelming apoptosis/necrosis, in which WPE significantly reverses these damaging processes. On KEGG analysis from heatmap (Fig. 4B), since WPE contained significant beneficiary fats, including mono- or polyunsaturated fatty acids as well as fat soluble vitamins, significant protective action implicated fat utilization was noted on KEGG analysis and on STRING analysis (Fig. 4C). On Fig. 5, we

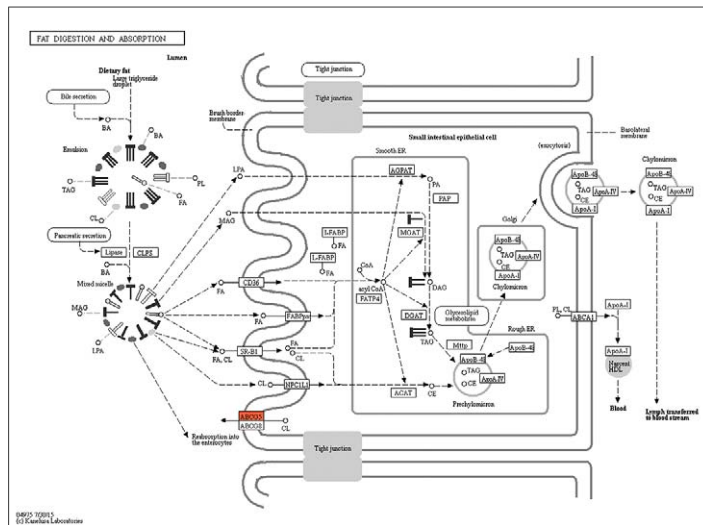
presented all gene status in bar analysis to compare AGS cells alone, AGS cells infected with *H. pylori*, and AGS cells infected with *H. pylori* in the presence of WPE. Conclusively, WPE significantly triggered to cope with damaging mechanisms of *H. pylori* infection.

Genes category significantly different that elevated after *H. pylori* infection, but further significantly elevated with WPE in the presence of *H. pylori* infection. Figure 6A showed heatmap comparing the genes of pooled samples featuring genes markedly increased, accentuated genes with WPE admin-

A Genes category different significantly which showed ↓ *H. pylori* infection, but ↑ with WPE in the presence of *H. pylori* (filtered 138 genes, $p < 0.05$)



B



C

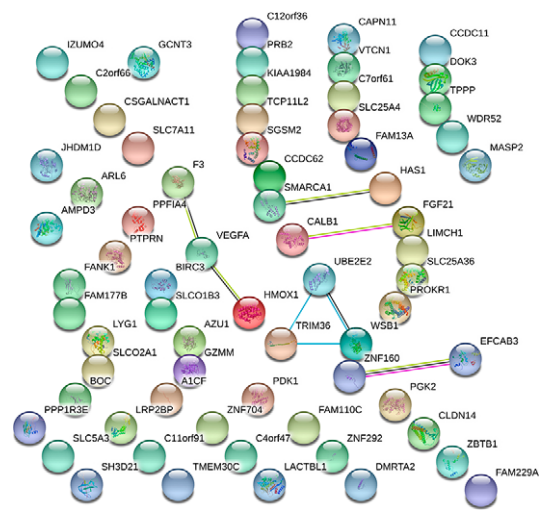


Fig. 4. (A) Expression heatmap for differential expressed genes showing significant trend like decreased with *H. pylori* infection, but significantly increased with co-treatment of WPE, description of gene category, GO enrichment analysis in box. (B) KEGG analysis Annotation of KEGG lipid-related signaling pathways with transcriptomic data. (C) STRING-generated interaction network of the differentially expressed gene showing amplification of the network for PPI associated with HMOX1 (heme oxygenase 1). See color figure in the on-line version.

istration among genes significantly increased after *H. pylori* infection, denoting WPE continue to increase against *H. pylori* infection, inferring restorative, anti-mutagenic, and rejuvenating genes in *H. pylori* infection, that is, *Ras small GTPase superfamily 3A* (RAB3A)/*RelB TNFAIP3-interacting protein 1* (RELBTNIP1)/*C17orf96/electron transfer flavoprotein subunit beta* (ETFEB)/*proto-oncogene isoform A2 of eukaryotic translation elongation factor eEF4* (EEF1A2)/*inositol phosphate 5-phosphatase J* (INPP5J)/*protein phosphatase methyltransferase 1* (PPME1)/*Ras homolog family member B* (RHOB)/*triosephosphate isomerase 1* (TPI1) as methylation implicated genes, *fos like 1*, *AP-1 transcription factor subunit* (FOSL1)/*NF-kappa-B heterodimeric RelB* (JUND.RELB) as response to cytokine, *kruppel-like factor 2* (KLF2)/*mucin 1* (MUC1) as positive regulation of transcription from RNA polymerase II promoter in response to stress, *n-Myc*

downstream regulated 1 (NDRG1)/*aldolase, fructose-biphosphate A* (ALDOA)/*enolase 1* (ENO1)/*phosphofructokinase* (PFKP)/PPME1 as cell-cell adhesion genes, and *glycosylphosphatidylinositol* (GPI)/*growth differentiation factor 15* (GDF15)/*neurturin* (NRTN) as growth factors. On STRING analysis, biological process such as canonical glycolysis, pyruvate metabolic process, nucleotide phosphorylation/pyridine-containing compound metabolic process and the regulation of cellular component organization (Fig. 6B–D). All discovered genes were listed in Fig. 7 showing enhanced expressions of genes with WPE in the presence of *H. pylori* infection.

Genes category significantly different that decreased after *H. pylori* infection, but further significantly decreased with WPE in the presence of *H. pylori* infection. Figure 8A showed heatmap comparing the genes of pooled samples featuring

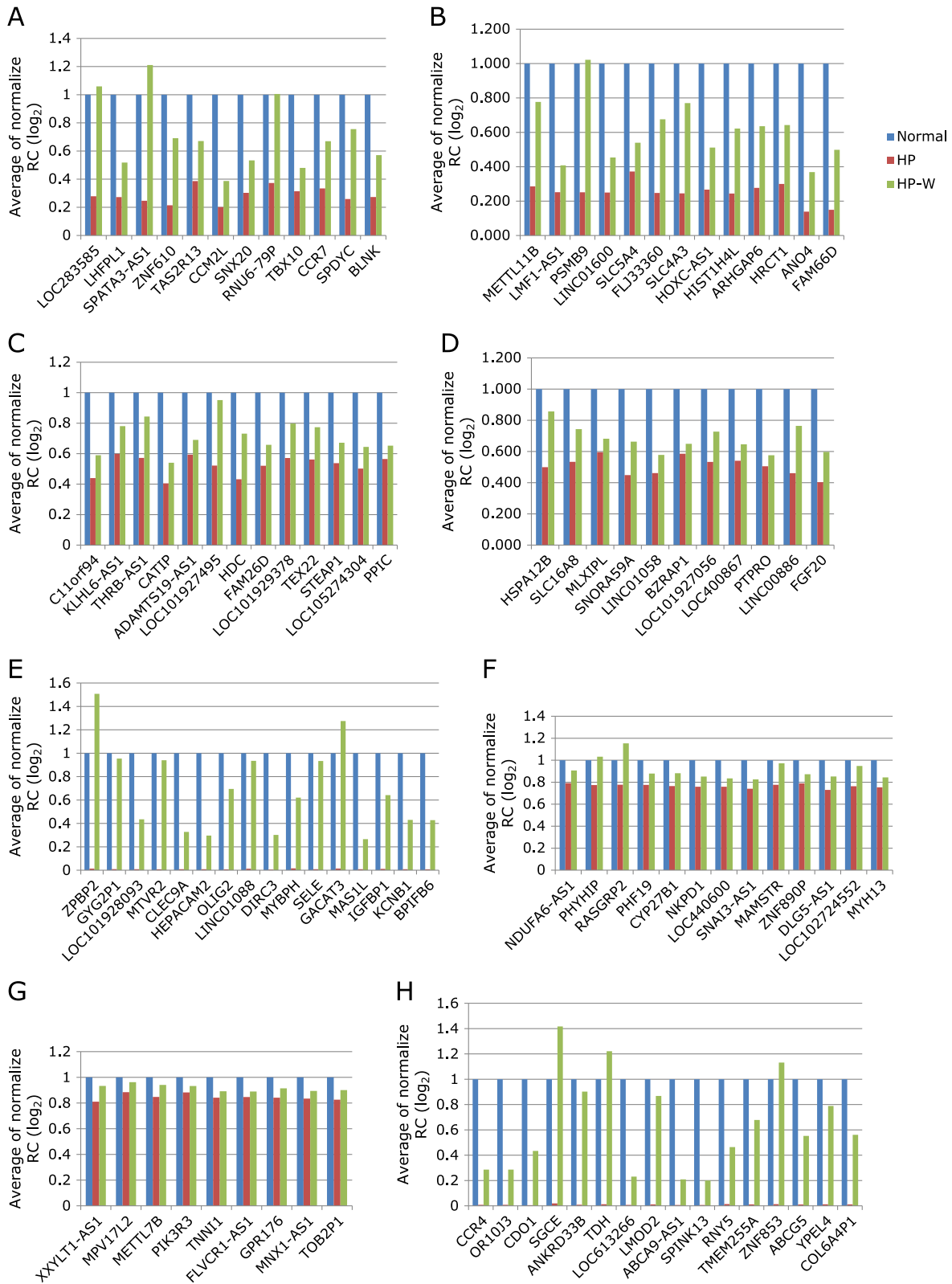


Fig. 5. Whole genes analyzed as significantly different, significantly decreased after *H. pylori* infection, but significantly increased after *H. pylori* infection in the presence of WPE ($p < 0.05$). HP, *H. pylori*; HP-W, *H. pylori* + WPE.

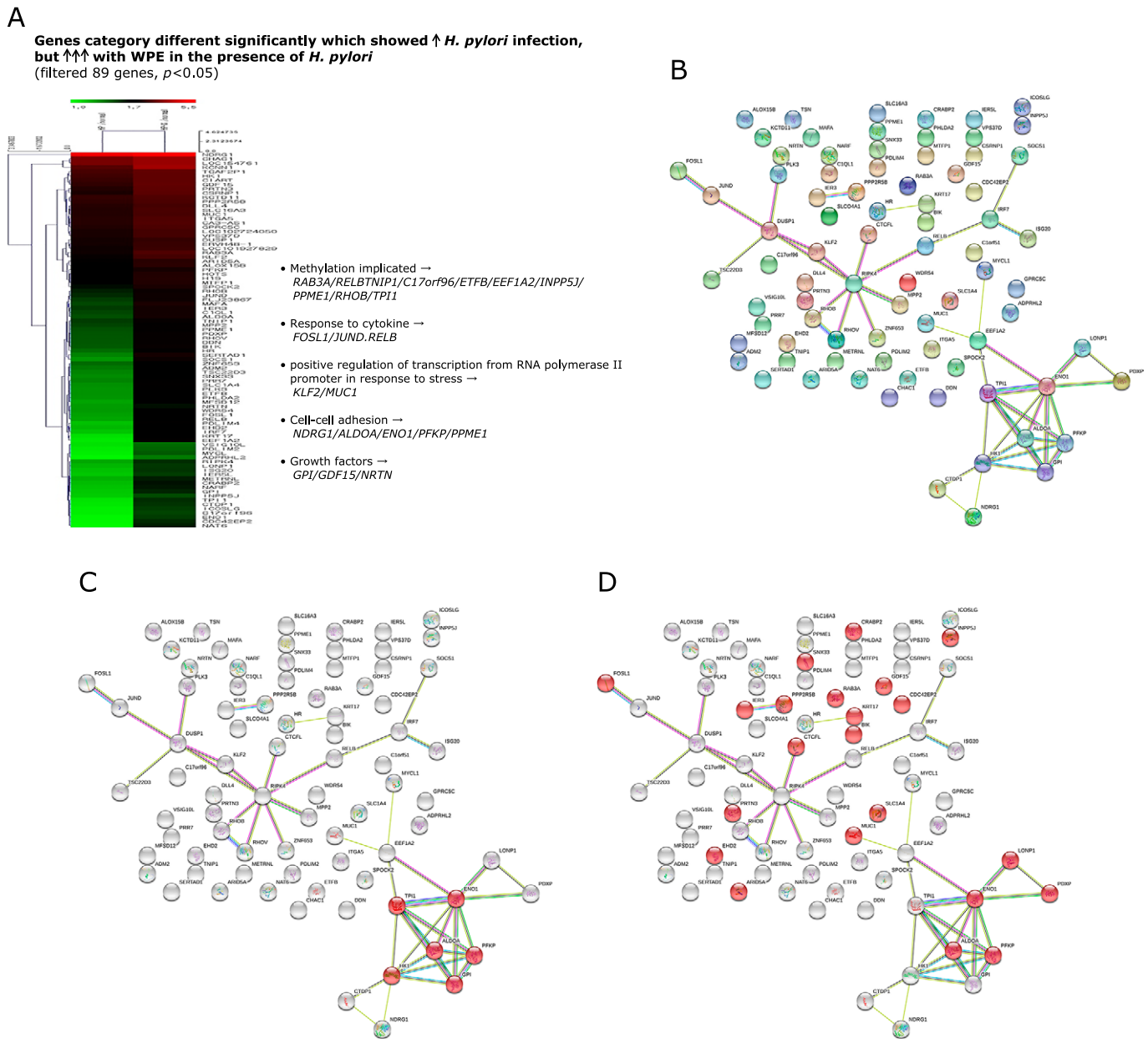


Fig. 6. (A) Expression heatmap for differential expressed genes showing significant trend like increased with *H. pylori* infection, but further significantly increased with co-treatment of WPE, description of gene category, GO enrichment analysis in box. (B–D) STRING-generated interaction network of the differentially expressed genes. See color figure in the on-line version.

genes markedly decreased genes with WPE administration among genes significantly decreased after *H. pylori* infection, denoting WPE continue to inhibit genes to prevent *H. pylori* infection-associated gastric damages and carcinogenesis, inferring cancer preventive in *H. pylori* infection. STRING analysis, significant cancer preventive and anti-mutagenesis was noted with WPE (Fig. 8B) and all discovered genes were listed in Fig. 9 showing enhanced expressions of genes with WPE in the presence of *H. pylori* infection, that is, *tetraspanin 32* (TSPAN32)/*HOXC cluster antisense RNA3* (HOXC-AS3)/*sarcoglycan gamma* (SGCG)/*spermatogenesis association 41* (SPATA41)/*HCG8 HLA complex group 8* (HCG8)/*butyrophilin like 8* (BTNL8)/*chemokine C-X-C motif ligand 3* (CXCL3).

Discussion

In the current study, we analyzed transcriptomic profiling of the influence of WPE administration on *H. pylori*-infected AGS cells through RNAseq analysis and determined that genes transcribed by WPE exerting defense and restorative genes as well as anti-inflammatory and anti-oxidative genes, by which we can further confirm our previous publications showing that dietary intake of walnut can be of anticipatory food factor to mitigate *H. pylori* infection (Fig. 10). As summarized in Fig. 10, transcriptomic profiling of genes relevant to walnut administration significantly guided the beneficiary biological actions as well as correcting pathogenic basis of *H. pylori* infection and to draw optimal biomarkers to guide clinical advises of food factor for troublesome *H. pylori* infection. In the current investigation, the

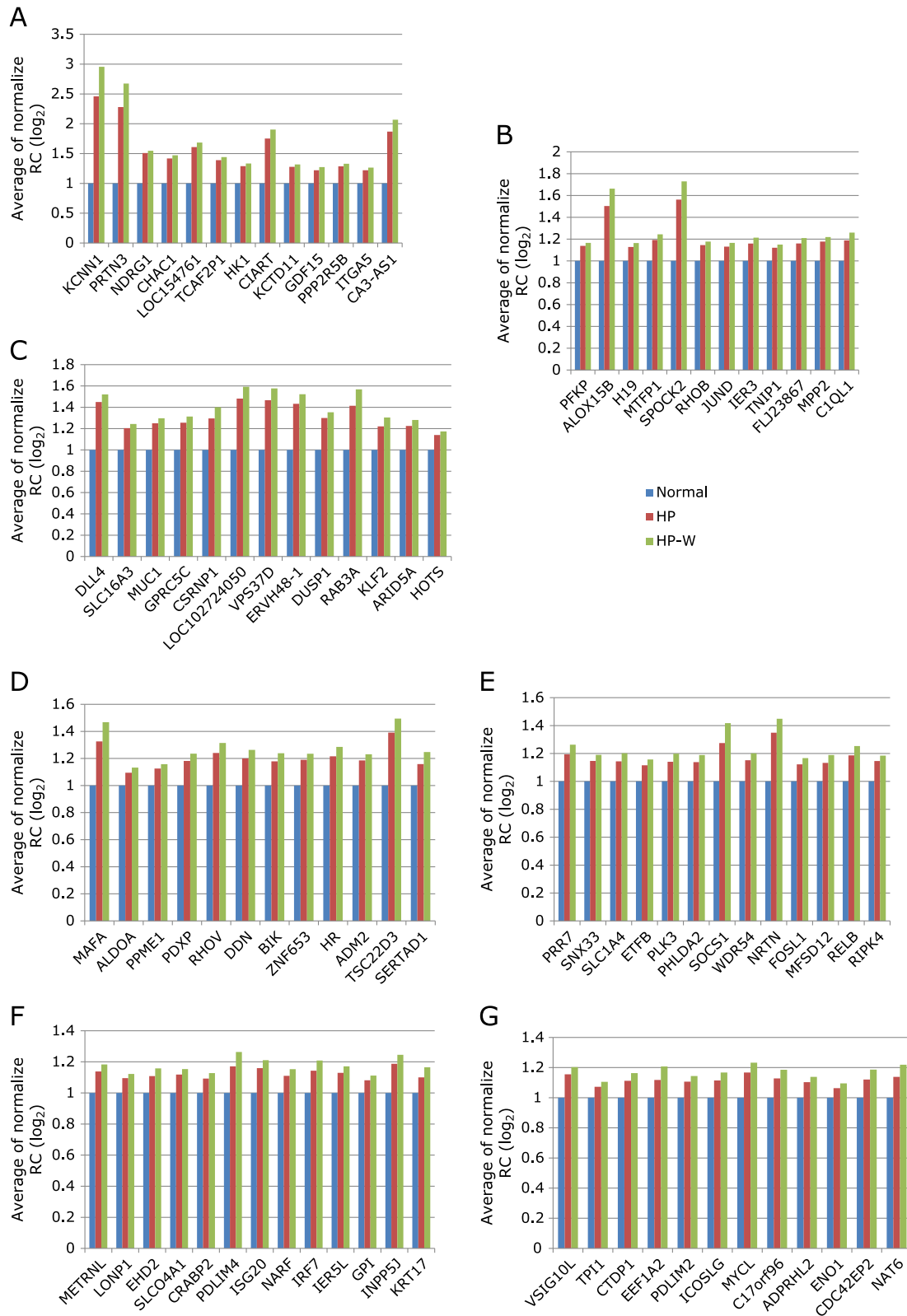
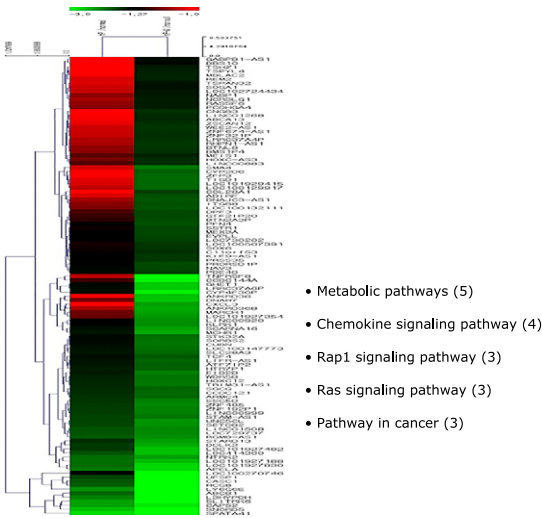


Fig. 7. Whole genes analyzed as significantly different, significantly increased after *H. pylori* infection, but further significantly increased after *H. pylori* infection in the presence of WPE ($p < 0.05$). HP, *H. pylori*; HP-W, *H. pylori* + WPE.

A

Genes category different significantly which showed ↓ *H. pylori* infection, but ↓↓↓ with WPE in the presence of *H. pylori* (filtered 89 genes, $p < 0.05$)



B

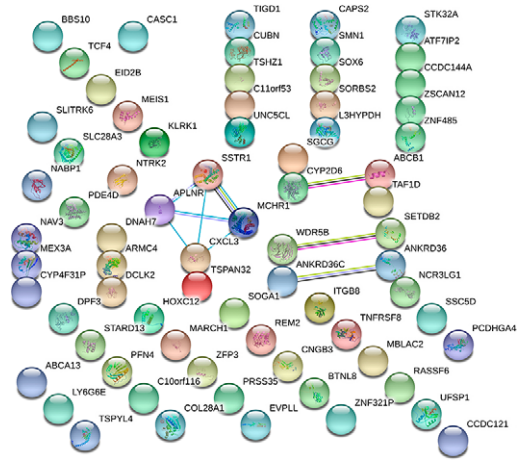


Fig. 8. (A) Expression heatmap for differential expressed genes showing significant trend like decreased with *H. pylori* infection, but further significantly decreased with co-treatment of WPE, description of gene category, GO enrichment analysis in box. (B) STRING-generated interaction network of the differentially expressed genes. See color figure in the on-line version.

following four points were highlighted relevant to walnuts efficacy against *H. pylori* infection, one was up-regulated transcriptomes after *H. pylori*, but significantly down-regulated with walnut, the second was down-regulated transcriptomes after *H. pylori*, but significantly up-regulated with walnut, signifying the conditions inferring mitigating damaging process, third was accentuating defense conditions by WPE and up-regulated after *H. pylori* infection, but further elevated with WPE, and the fourth was ameliorating offense conditions by *H. pylori* infection, but further elevated with WPE to enforce anti-damaging condition, signifying condition inferring restorative and anti-mutagenic process with WPE.

For the current investigation, RNAseq is the use of NGS technology to sequence cDNA (reversed transcribed from RNA) in order to obtain information about RNA. Compared to microarray technology, RNAseq technology offers several obvious advantages, 1) allowing the detection of all isoforms of a gene, even novel ones because microarray only relies purely on previous knowledge regarding genes to design probes for detection, 2) revealing microarray usually on the gene and exon level, whereas RNAseq can reach to the level of a single nucleotide, 3) allowing detection of single nucleotide variance and structural variants, such as small insertions, deletions, alternative splicing, and gene fusion.⁽³²⁻³⁴⁾ Taken briefly, RNAseq is a recently developed method for transcriptome profiling that employs NGS technologies and measures transcript levels with increased precision compared with other approaches.⁽³⁵⁾

First of all, genes including *CCR4* and *CCR* as chemokine receptor family gene, *BLNK*, *CCR7*, *CCR4*, *CDO1*, *KLRG1*, *SELE* as inflammatory response gene, and *CCR4*, *CCR7*, *RASGRP2*, and *PIK3R3* as chemokine signaling pathway related genes were explored through down-regulating genes by WPE in the presence of *H. pylori* infection. Looking at each gene with additional identified genes as presented in Fig. 3, WPE seems to be beneficial as damage-reducing food factor. During *H. pylori* infection, different kinds of inflammatory cells such as dendritic cells, macrophages, neutrophils, mast cells, eosinophils, T cells, and B cells are accumulated into the stomach, during which pathologic

process, the interactions between chemokines and their respective receptors recruit particular types of the leukocytes that ultimately determine the nature of immune response and ensuing gastric pathologies. The chemokines epoch shapes immune response in the early stages of *H. pylori* infection shapes in order to eliminate the *H. pylori*.⁽³⁶⁾ Thinking the link between chronic *H. pylori* infection and gastric carcinogenesis, resolution of gastric cancer-promoting inflammation can be novel strategy for anti-cancer therapy, walnut in the current investigation.^(18,37) Similar results regarding anti-*H. pylori* infection with antioxidative astaxanthin via transcriptome analysis was reported⁽³⁸⁾ that astaxanthin significantly counteracted *H. pylori*-induced activation of PI3K and phospholipase- γ , concluding that astaxanthin treatment suppressed *H. pylori*-induced gastric cancer progression by inhibiting cytoskeleton reorganization and reducing cell motility through downregulation of PI3K as well as *c-MET*, *EGFR*, *PLC γ 1*, *Cdc42*, and *ROCK1*. Also, STRING analysis showed PPI centered PI3K in this inhibitory action of WPE against *H. pylori* infection since adrenomedullin production was promoted via PI3K-AKT signaling pathway activation by gastric epithelial cells in *H. pylori cagA*-dependent manner, and resulted in increased inflammation within the gastric mucosa, additionally *H. pylori* infection biofilm involves a multigene stress-biased response including *H. pylori* flagella, identified through RNAseq analysis.⁽³⁹⁾ As well as *H. pylori* infection, chronic gastrointestinal ischemia is the result of decreased mucosal perfusion, in which hypoxia-inducible factor-1 α (HIF-1 α) is expressed highly⁽⁴⁰⁾ and is regarded as core mechanisms for *H. pylori* infection relevant gastritis as well as gastric cancer.⁽⁴¹⁾

Next, *BIRC3* and *SLC25A4* as regulatory genes of necroptotic process, *f3* transcription, *VEGFA*, and *AZU1* genes as positive regulation of positive chemotaxis, and *HMOX1* as positive regulation of angiogenesis were explored through up-regulating genes by WPE in the presence of *H. pylori* infection. Heme oxygenase-1 (HO-1) is the rate-limiting enzyme in the catabolism of heme, followed by production of biliverdin, free iron and carbon monoxide as a stress-responsive protein induced by various oxidative agents including *H. pylori* infection.⁽⁴²⁾ There-

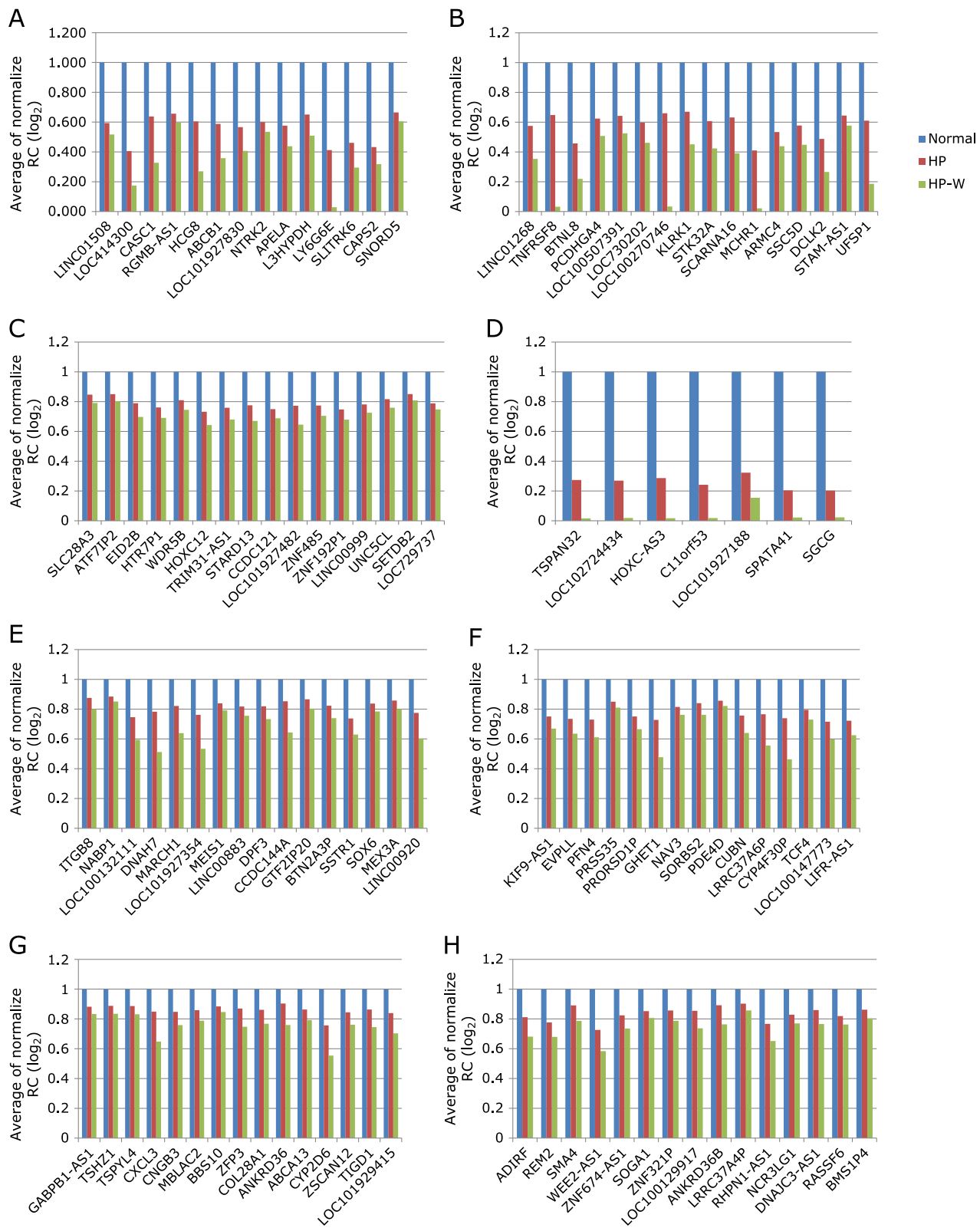


Fig. 9. Whole genes analyzed as significantly different, significantly decreased after *H. pylori* infection, but further significantly decreased after *H. pylori* infection in the presence of WPE ($p < 0.05$). HP, *H. pylori*; HP-W, *H. pylori* + WPE.

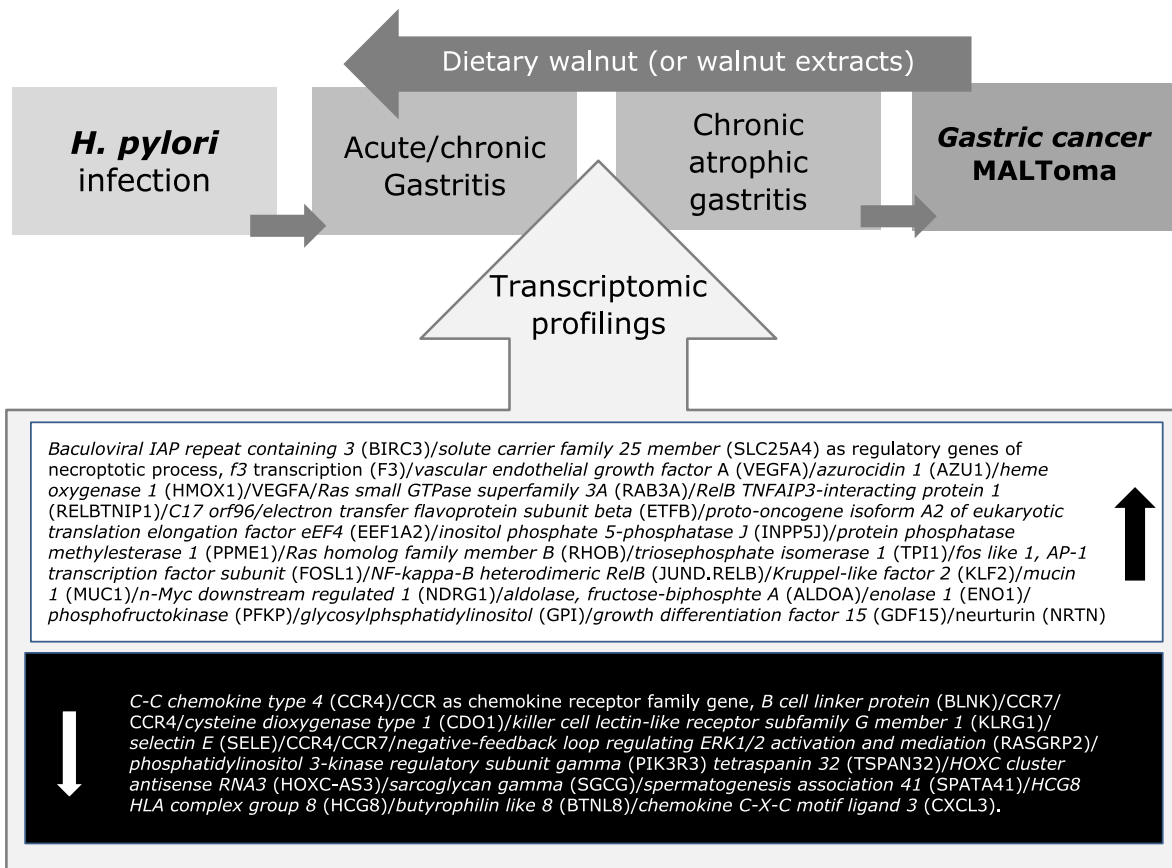


Fig. 10. Schematic presentation showing the transcriptomic profiling showing genes implicated in rescuing action of WPE against *H. pylori* infection.

fore, the induction of HO-1 expression plays a critical protective role in intestinal damage models induced by ischemia-reperfusion damages, non-steroidal anti-inflammatory drugs (NSAID)-induced gastrointestinal damages, trinitrobenzene sulfonic acid or dextran sulfate sodium-induced colitis as well as stomach lesion.^(43,44) HO-1 inhibits IL-8 secretion by *H. pylori*-infected cells, by which the innate immune response of the host can restrict the pathogenicity of *H. pylori* as well as imposing significant rescuing action against *H. pylori*-associated oxidative stress.⁽⁴⁵⁾ Taken together with transcriptomic profiling explored relevant to walnut in the presence of *H. pylori* infection, walnut afforded either significant protective or anti-inflammatory benefits-relevant genes.

In addition, we could further explored genes, increased after *H. pylori* infection, but further up-regulated with WPE in the presence of *H. pylori* infection (Fig. 6A) and *vice versa* (Fig. 8A), among which denoting ultimate transcriptome profiling such as *RAB3A*, *NRLBTNIP1*, *ETFB*, *EEF1A2*, *INPP5J*, *PPME1*, *RHOB*, and *TPI1* as methylation implicated genes, *FOSL1* and *JUND.RELB* as response to cytokine, *KLF2* and *MUC1* as positive regulation of transcription from RNA polymerase II promoter in response to stress, *NDRG1*, *ALDOA*, *ENO1*, *PFKP*, and *PPME1* as cell-cell adhesion genes, and *GPI*, *GDF15*, and *NRTN* as growth factors. Among transcriptomes, growth factors related genes such as *GPI*, *GDF15*, *NRTN*, *Muc1*, *KLF2* are attractive because these are intervened into wound healing, though cancer is regarded as abnormal wound healing. Retarded and repeated healing disturbance can explain pathogenesis of *H. pylori*-associated gastroduodenal ulceration as well as gastric cancer.⁽⁴⁶⁻⁴⁸⁾ Since the cytoplasmic tail domain of cell surface mucins, for instance, *Muc1*, are capable of initiating signal transduction cascades and

may play an important biological role in cellular signaling as a protective barrier and regulating inflammation and inflammasome against *H. pylori*.^(49,50) Also, methylation phenotype and rapid demethylation has been regarded as crucial tumorigenesis of *H. pylori* infection, by which wise action of WPE can be core mechanisms.⁽⁵¹⁾

Lastly, transcriptome genes including *TSPAN32*, *HOXC-AS3*, *SGCG*, *SPATA41*, *HCG8*, *BTNL8*, *CXCL3*, etc. were identified as genes down-regulated with *H. pylori* infection, but further decreased in the presence of WPE. Focused on *CXCL3*, according to our study,⁽⁵²⁾ we have found that *H. pylori CagA* significantly suppressed TGF- β /*Smad* transcriptional responses through critical inhibition of *Smad3*, though *CagA* interacted constitutively with *Smad2*, *Smad3*, and *Smad4* and *H. pylori CagA* significantly inhibited TGF- β -induced suppression of proinflammatory chemokines including *IL-8*, *CXCL1* and *CXCL3*. Also, *HOXC* clusters gene had been regarded as oncologic factor, especially in gynecology cancer.⁽⁵³⁾ Butyrophilin-like receptor (*BTNA*), though only studied in pancreatic adenocarcinoma,⁽⁵⁴⁾ macrophage-related diseases,⁽⁵⁵⁾ and uveal melanoma,⁽⁵⁶⁾ can be identified as down-regulated target of WPE after current investigation.

Our and other investigators^(1,4,18,57-59) significantly suggested that the food factor may play a critical role in determining the final outcome of *H. pylori* infection particularly if certain intake of dietary food factor, walnut in this investigation, 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. In this effort, the current high throughput analysis, RNAseq transcriptome profiling, provides definite advantage and usefulness to explain

beneficiary action of walnut as well as potential as pharmaceutical for *H. pylori* infection.

Author Contributions

Study concept and design: JMP and KBH; acquisition of data: JMP, YMH, HJL, and SJH; analysis and statistical analysis: JMP and KBH; interpretation of data: SJK and KBH; drafting of manuscript: KBH.

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Abbreviations

CAG	chronic atrophic gastritis
CXCL3	chemokine C-X-C motif ligand 3
GO	gene ontology
HMOX 1	heme oxygenase-1
HTP	high throughput analysis
<i>H. pylori</i>	<i>Helicobacter pylori</i>
MOI	multiplicity of Infection
NGS	next generation sequence
STRING	Search Tool for the Retrieval of Interacting Genes/Proteins
RNaseq	RNA sequencing

Conflict of Interest

No potential conflicts of interest were disclosed.

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