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Analysis of Gene Expression Profile of AGS Cells Stimulated by *Helicobacter pylori* Adhesion

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Background/Aims: Interactions between *H. pylori* and gastric epithelial cells contribute to gastric inflammation and epithelial damage. This study was performed to evaluate the gene expression profile of AGS cells by adhesion of *H. pylori*. **Methods:** Changes in AGS cell gene expression induced by co-culturing with *H. pylori* (G69a strain) (4, 12, 24, 48 hours) were monitored using oligonucleotide microarray. Real-time reverse transcription-polymerase chain reaction (RT-PCR) was performed for data validation by the Assay-on-Demand Gene Expression product method. **Results:** A total of 270 (2.66%) and 19 genes (0.19%) were up-regulated in AGS cells by *H. pylori* adhesion. Gene ontology analysis showed that up-regulated genes were categorized into endolipidase activity (17 genes), receptor binding (17 genes), integrin binding (4 genes), and two down-regulated genes into GTP binding category. The expression levels of 20 up- and 5 down-regulated genes were quantified by real-time RT-PCR. Sixteen genes involving cytokine activity (IL8, IL1B, TNF), hydrolase activity (PTP4A1, ERCC1, CASP8, CASP7, ACIN1), VIP receptor activity (VIPR2), and neuropeptide Y receptor activity (GPR83) were confirmed to be up-regulated. Five genes, namely, ARF3, M17S2, DDB2, AWP1, and WTAP were confirmed to be down-regulated. **Conclusions:** Host genes are significantly changed by *H. pylori* adhesion, which might explain the gastroduodenal pathogenesis induced by *H. pylori* infection. **(Gut and Liver 2007;1:40-48)**

Key Words: *Helicobacter pylori*; Host cell; Adhesion; Microarray

INTRODUCTION

Helicobacter pylori (H. pylori) is a non-invasive, gram-negative bacterium that colonizes in gastric mucosa. Gastric colonization by *H. pylori* results in a mucosal inflammatory response. It is a risk factor for peptic ulcer disease and gastric malignancy. The elucidation of the mechanism underlying the inflammation and damage of underlying tissue would be important in the understanding of the development and progression of these gastro-intestinal disorders.

The fact that less than 20% of infected individuals present with clinical disease, suggests that disease severity is dependent on the interaction between the pathogen and the host.[1] Moreover, this host-pathogen interaction is likely to induce changes in gene expression in both the host and *H. pylori*. Previously, by using a macroarray method, we found that 22 and 21 *H. pylori* genes were consistently up- or down-regulated, respectively by adhesion to AGS cells.[2] Moreover, these alterations in *H. pylori* gene expression in vitro suggested that changes of motility, outer membrane composition and stress responses might be involved in gastric colonization.[2] This study was performed to evaluate the gene expression profile in AGS cells by adhesion of *H. pylori* by microarray method.

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MATERIALS AND METHODS

1. Bacterial strain

H. pylori strain G69a ($CagA^+$, $VacA^+$) expressing green fluorescence protein (GFP) (a gift from Dr. Reiner Haas, Munich, Germany) was used to observe the attachment of *H. pylori* to the human gastric cancer cell line, AGS cells (ATCC CRL 1739). Bacteria were grown for 16 hour under micro-aerobic conditions (5% O_2, 10% CO_2 and 85% N_2) at 37°C on trypticase soy agar (TSA) II plates supplemented with 5% sheep blood. Bacteria were harvested and resuspended in RPMI 1640 medium (BRL/Life Technologies Inc., Gaithersburg, MD) for co-culture with AGS cells.

2. Co-culture conditions

AGS cells were grown to confluence in tissue culture flasks. AGS monolayers were washed twice with RPMI 1640 medium, and incubated with *H. pylori* at a multiplicity of infection (MOI) of 100:1 at 37°C in a micro-aerobic atmosphere for 4, 12, 24, and 48 hours. Non-infected flask of AGS cells served as a negative control. *Hp*/AGS co-cultures were washed twice with RPMI 1640 to remove unattached *H. pylori* and debris after 1 hour incubation, and further incubated for an additional time. At the end of incubation period *Hp*/AGS co-culture was washed again two times with phosphate-buffered saline (PBS, pH 7.4, 37°C) to remove any unattached *H. pylori*. AGS control flasks were treated in the same manner.

3. RNA extraction and fluorescence labeling

Total RNA was extracted with TRIzol® reagent (Invitrogen, Carlsbad, CA, USA) from each group of cells, as described by the manufacturer. Collected RNAs were purified using RNeasy mini kits (Qiagen Inc., Valencia, CA, USA). RNA samples were then treated with DNase I (Invitrogen) and RNA levels were quantified by spectrophotometry and by using an Agilent 2100 Bioanalyzer with RNA 6000 Nano Assay kits (Agilent Technologies, Mountain View, CA, USA). Total RNA was amplified and then labeled in the presence of fluorescent dNTP (Cy3 dUTP or Cy5 dUTP, GE Healthcare, Piscataway, NJ, USA).

4. Oligonucleotide microarray

In the present study, we used Macrogen Human Oligo 10K Chip (Macrogen, Seoul, Korea), as described previously.[3] Cy-labelled total RNA from untreated AGS cells were used as a reference in all experiments. Total RNAs from each group of cells were hybridized with a reference in quadruplicated biological replicates including one dye swapping. Cy3 and Cy5 fluorescent intensities were determined using the GenePix scanner (Axon Instruments, Union City, CA, USA), and images were analyzed using the GenePix Pro to calculate relative ratios and to determine confidence intervals.

5. Data analysis

Fluorescence intensities were measured using GenePix Pro software and processed by the variance stabilizing normalization method.[4] After performing intensity-dependent global LOWESS regression, spatial and intensity dependent effects were managed by pin-group LOWESS normalization.

6. Data validation by real-time RT-PCR

A selection of genes that showed altered expression in AGS cells by microarray analysis was re-examined by real-time reverse transcription-polymerase chain reaction (RT-PCR) to confirm the changes observed. First, cDNA was generated from 1 g of total RNA and PCR cycling was performed on a 7000HT Sequence Detection System in 25 l SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) using 25 ng of cDNA. Target genes were tested using Assay-on-Demand Gene Expression products. Primer design was ordered according to http://www.appliedbiosystems.com/. The homo sapiens beta-actin gene was used as an endogenous reference to control for expression independent sample-to-sample variability. Relative gene expressions were determined from the obtained Ct values and using the $2^{-\Delta\Delta Ct}$ method.[5] All equipment and reagents were purchased from Applied Biosystems and used according to their protocols. However, in the case of B1 for mucin, its up-regulation was confirmed by using the rapid construction of cRNA standard curves in quantitative real-time RT-PCR[6] because there was no adequate primer for B1 for mucin among the Assay-on-Demand (AOD) Gene Expression products. In this case, human beta-actin gene was also used as an endogenous reference. The following primers were used: B1 for mucin forward, TCACTTGACCCAGGAGGTGG; B1 for mucin reverse, TCTTGCTCTGTTGCCAGGCTA; Homo sapiens actin, beta forward, TTCGAGCAAGAGATGGCCAC; and Homo sapiens actin, beta reverse, CGGATGTCCACGTCACACTT. IL8 was confirmed by both methods (the AOD Gene Expression product method and the rapid construction of cRNA standard curves in quantitative real-time RT-PCR method) and the results obtained using both methods were similar.

RESULTS

1. Regulation of gene expression in AGS cells after *H. pylori* adhesion

The attachment of *H. pylori* to AGS cells has been already characterized by observing the *Hp*/AGS co-culture in the z axis by confocal microscopy.[2] All the GFP-containing organisms remained associated with the AGS cells after two washing steps, and thus, AGS cells that had adhesion with *H. pylori* were compared with AGS cells. To confirm that cDNAs hybridized to the array were derived from AGS RNA, and not from *H. pylori*, an equal amount of RNA from *H. pylori* was examined using a Human Oligo 10K Chip, and no signals were shown by slides hybridized with the cDNA of *H. pylori* alone (data not shown), indicating that the chip cDNA hybridization was specific for eukaryotic cells. When a chip of *Hp*/AGS co-culture was compared with one of the normal AGS cells, 270 genes were found to be enhanced by more than 2-fold, and 19 genes were found to be attenuated by more than 2-fold at least in one time after 4, 12, 24, and 48 hour of incubation or at least in three times of four

Table 1. Gene Ontology (GO) Analysis of Genes Up-regulated in AGS Cells Co-cultured with *H. pylori*

GO ID	GO category	Genes	No. of genes	p-value
GO:0004175	Endopeptidase activity	ADAM11, ADAM17, ADAM22, ADAMTS8, CAPN9, CASP3, CASP7, CASP8, CORIN, KLK3, PRSS1, PSMB10, TLL2, USP3, USP7, USP18, USP32	17	0.0000174
GO:0005102	Receptor binding	ADAM11, ADAM22, ADAMTS8, DMP1, FGF2, GPI, GRN, IFNA8, IL1B, IL1F5, IL8, PROK1, SMARCD3, SORBS1, TGFA, TNF, VEGFC	17	0.0000384
GO:0005178	Integrin binding	ADAM11, ADAM22, ADAMTS8, DMP1	4	0.000126
GO:0005315	Inorganic phosphate transporter activity	ADAMTS8, SLC25A3, SLC34A1	3	0.000172
GO:0003677	DNA binding	ACIN1, ASCL2, CARHSP1, CHC1, CREB5, ELF5, ERCC1, ETS2, HIST1H1A, HMGN1, HOXA13, HSF1, IRF5, MAF, MAFF, MAFG, MBD1, MNAB, MRE11A, MXD4, NR1D2, NR2E1, OGG1, PAX9, PITX1, POLR3F, POU3F1, RAI1, RBL1, REL, SIM2, SMARCA2, SOX1, SRCAP, TAF7L, TCF19, THRA, WT1, ZBTB7, ZIC1, ZNF24, ZNF145, ZNF192, ZNF354C	44	0.000209
GO:0008083	Growth factor activity	FGF2, GPI, GRN, IL1B, IL1F5, PROK1, TGFA, VEGFC	8	0.000547
GO:0030693	Caspase activity	CASP3, CASP7, CASP8	3	0.00198
GO:0005125	Cytokine activity	GPI, GRN, IFNA8, IL1B, IL1F5, IL8, TNF	7	0.00555
GO:0030695	GTPase regulator activity	ARFGEF1, ARHGEF3, BCAR3, CHC1, DDEF2, MCF2, PLCE1, RASA1, RGS9	9	0.00938
GO:0030247	Polysaccharide binding	ADAMTS8, CD44, FBLN1, FGF2	4	0.00978
GO:0003707	Steroid hormone receptor activity	NR2E1, NR1D2, THRA	3	0.0293
GO:0008509	Anion transporter activity	ADAMTS8, CLCN3, SLC25A3, SLC4A2	4	0.0338
GO:0008047	Enzyme activator activity	DDEF2, DBNL, IGFBP3, RASA1, RGS9	5	0.0606

Table 2. Gene Ontology (GO) Analysis on Genes Down-regulated in AGS Cells Co-cultured with *H. pylori*

GO ID	GO category	Genes	No. of genes	p-value
GO:0005525	GTP binding	ARF3, GTPBP4	2	0.0437
GO:0003677	DNA binding	AWP1, CSDA, MYF5, TRAF4	4	0.0947
GO:0005515	Protein binding	CNTN2, FAF1, PACSIN1, PCDHA1	4	0.106
GO:0008083	Growth factor activity	GDNF	1	0.114
GO:0004252	Serine-type endopeptidase activity	CMA1	1	0.142
GO:0043169	Cation binding	M17S2, TRAF4	2	0.254
GO:0004672	Protein kinase activity	PACSIN1	1	0.432
GO:0005509	Calcium ion binding	PCDHA1	1	0.449

Table 3. Gene Ontology (GO) Analysis on the Confirmation of Genes Up-regulated in AGS Cells by *Hp*/AGS Co-culture

GO ID	GO Category	Genes	No. of genes	p-value
GO:0030693	Caspase activity	CASP7, CASP8	2	0.000138
GO:0005125	Cytokine activity	IL1B, IL8, TNF	3	0.000398
GO:0004999	Vasoactive intestinal polypeptide receptor activity	VIPR2	1	0.00363
GO:0004983	Neuropeptide Y receptor activity	GPR83	1	0.00784
GO:0004089	Carbonate dehydratase activity	CA1	1	0.018
GO:0003684	Damaged DNA binding	ERCC1	1	0.0339
GO:0016787	Hydrolase activity	ACIN1, CASP7, CASP8, ERCC1, PTP4A1	5	0.0423
GO:0004725	Protein tyrosine phosphatase activity	PTP4A1	1	0.0753

experiments.

To characterize enhanced genes, we analyzed the gene ontology (GO) categories of the genes that were differentially expressed in *Hp*/AGS co-cultures.[7] The 270 genes up-regulated in *Hp*/AGS co-cultures were classified according to endopeptidase activity, receptor binding, integrin binding, inorganic phosphate transporter activity, and DNA binding (Table 1). On the other hand, the 19 down-regulated genes in Hp/AGS co-cultures were classified according to GTP binding, DNA binding, protein binding, growth factor activity, and serine-type endopeptidase activity (Table 2).

2. Confirmation of gene up- or down-regulation in AGS cells in Hp/AGS co-cultures by real-time RT-PCR

Of the 270 up- and 19 down-regulated genes, we selected 20 up- and 5 down-regulated genes according to priority of its importance to confirm the expression level difference by real-time RT-PCR using AOD Gene Expression products (n=5). Sixteen genes were confirmed to be up-regulated (Fig. 2), and 11 of theses were classified by GO category with significance (Table 3). Namely, genes were classified to caspase activity (CASP8, CASP7), cytokine activity (IL1B, IL8, TNF), vasoactive intestinal polypeptide receptor activity (VIPR2), neuropeptide Y receptor activity (GPR83), carbonate dehydratase activity (CA1), damaged DNA binding (ERCC1), hydrolase activity (ACIN1, ERCC1, PTP4A1), and protein tyrosine phosphatase activity (PTP4A1). In addition, TNFRSF21, CTNNBL1, PTPRCAP, UBL3 and B1 were also confirmed to be up-regulated by real-time RT-PCR. Of these 16 genes, the up-regulation of cytokine activity genes such as interleukin-1b (IL1B), interleukin 8 (IL8), and tumor necrosis factor (TNF superfamily, member 2) (TNF) were distinguished (Fig. 1). In case of IL1B, it reached 60.1-fold after 24 hours of co-culture (Fig. 2). Of the 20 selected up-regulated genes, DNBL and CINP were not confirmed by real-time RT-PCR. In cases of MAF and ZNF145, there was no PCR product obtained using AOD primers in either cDNA from *Hp*/AGS co-culture or cDNA from AGS alone. One possible explanation is that the level of expression of these two genes might be too low to precede for the confirmation of the work.

All 5 down-regulated genes selected were confirmed. ARF3 (ADP-ribosylation factor 3) (Fig. 3B), M17S2 (membrane component, chromosome 17, surface marker 2 (ovarian carcinoma antigen CA125) (Fig. 3C), DDB2 (damage-specific DNA binding protein 2, 48 kDa) (Fig. 3D), AWP1 (protein associated with PRK1) (Fig. 3E), and WTAP (Wilms' tumor 1-associating protein) (Fig. 3F) demonstrated down-regulation in co-culture with AGS versus human beta-actin, the housekeeping gene (Fig. 3A).

DISCUSSION

H. pylori is a pathogen that colonizes in human stomach. Understanding its pathogen-host interactions at the gene level could improve our understanding of the roles of host and organism during pathogenesis. The availability of cDNA micro-arrays for the ORFs of man and *H. pylori* means that global gene analysis is now possible. Whether fabricated as a slide microarrays or high-density oligonucleotide arrays or nylon membrane macro-array, analytical tools provide a basis for analyzing human or *H. pylori* gene expressional changes in cells caused by *H. pylori* adhesion. Functional genomics uses the expression profiling of mRNA to provide a condition-specific and time-specific genome-scale snapshot of the transcriptome. Several studies[1,8-13] have characterized host cell response to attached *H. pylori*, but each of these studies used different transformed cell lines [MKN 45 cells,[1] Kato 3 cells,[8] and AGS cells[9]] or a murine model,[10] and chips containing up to 57,800 genes[8] or specific functional genes, such as, 352 cancer-related genes.[9] Among these studies, there were few confirmation study on up- or

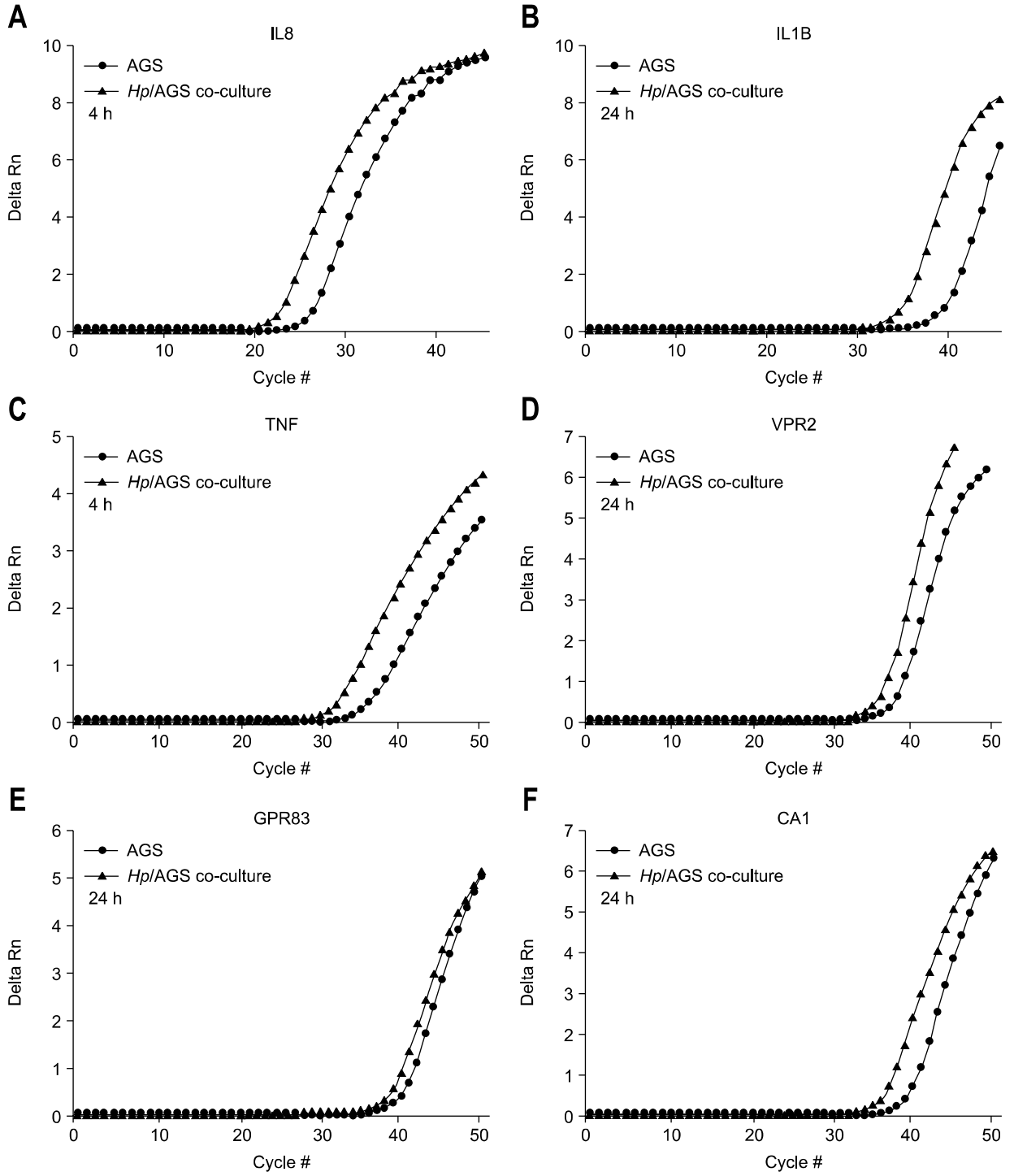

Fig. 1. Real-time RT-PCR of up-regulated genes. Up-regulations of (A) Interleukin 8 (IL8), (B) Interleukin 1b (IL1B), (C) Tumor necrosis factor (TNF) receptor superfamily member 21, (D) vasoactive intestinal peptide receptor 2 (VIPR2), (E) G protein-coupled receptor 83 (GPR83), and (F) carbonic anhydrase 1 (CA1) are confirmed by real-time RT-PCR. When cDNA encoding IL8, IL1B, TNF, VPR2, GPR83, or CA1 is amplified by real-time RT-PCR, these six genes are up-regulated with *H. pylori* co-culture.

down-regulated genes in the microarray experiment or on serial changes of gene expression depending on incubation time (4, 12, 24, 48 hour) of *Hp*/host cell co-cultures which were evaluated in the present study. Usually, there were huge amount of information from microarray studies that were difficult to isolate important information, systematically.

In this study GO analysis was used to determine the

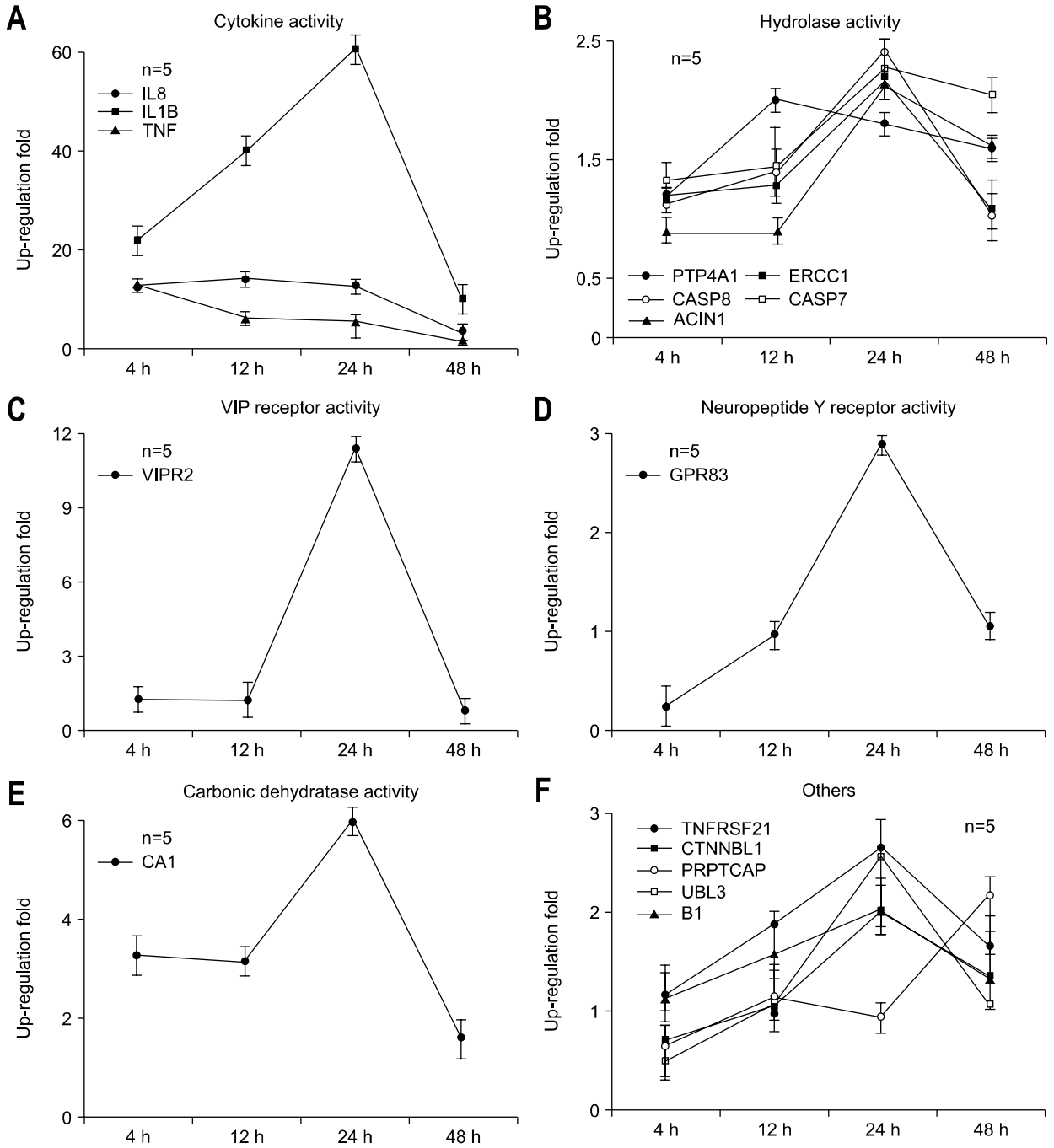

Fig. 2. Confirmation of genes up-regulated in *Hp*/AGS co-culture by real-time RT-PCR using Assay-on-Demand gene expression products at incubation times of 4, 12, 24, and 48 hours. When cDNA encoding 16 genes derived from AGS alone or *Hp*/AGS co-cultures are amplified by using real-time PCR and classified according to gene ontology (GO) categories, they are classified as follows; cytokine activity [IL8, interleukin 8; IL1B, interleukin-1b; TNF, tumor necrosis factor (superfamily, member 2)], hydrolase activity (PTP4A1, protein tyrosine phosphatase type IVA, member 1; ERCC1, excision repair cross-complementing rodent repair deficiency, complementation group 1; CASP8, caspase 8, CASP7, caspase 7; ACIN1, apoptotic chromatin condensation inducer in the nucleus), VIP receptor activity (VIPR2, vasoactive intestinal peptide receptor 2), neuropeptide Y receptor activity (GPR83, G protein-coupled receptor 83), carbonic dehydratase activity (carbonic anhydrase 1), and other categories (TNFRS21, tumor necrosis factor receptor superfamily, member 21; CTNNBL1, catenin, beta like 1; PRPTCAP, protein tyrosine phosphatase, receptor type, C-associated protein; UBL3, ubiquitin-like 3; B1, B1 for mucin). Data shown are the averages of five experiments with error bars indicating statistical errors of the mean value.

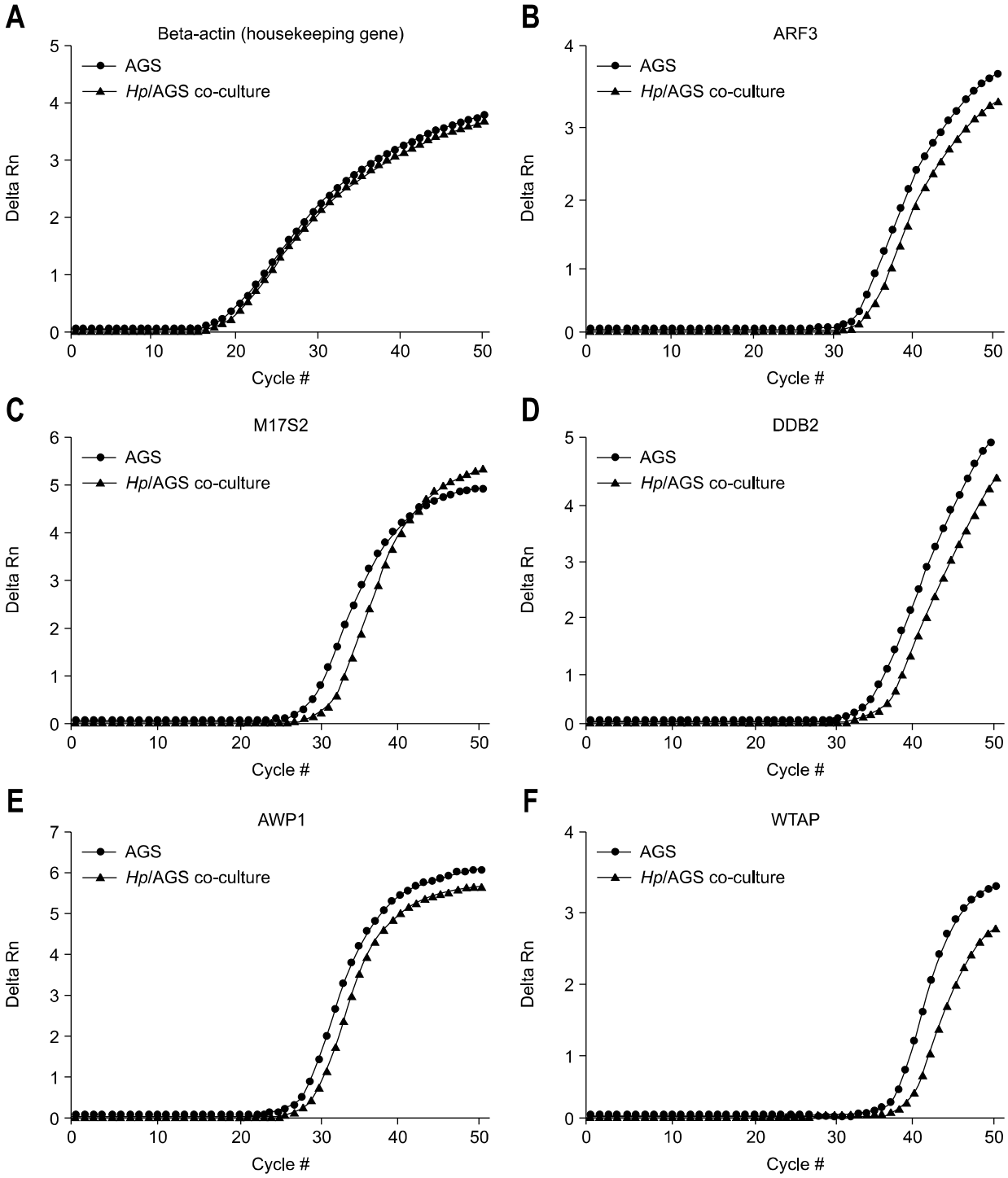

Fig. 3. Confirmation of 5 genes down-regulated in *Hp*/AGS co-culture. (B) ARF3, ADP-ribosylation factor 3, (C) M17S2, ovarian carcinoma antigen CA 125, (D) DDB2, damage-specific DNA binding protein 2, 48kDa, (E) AWP1, protein associated with PRK1, and (F) Wilms' tumor 1-associating protein (WTAP) are confirmed to be down-regulated.

functional classifications of up- and down-regulated genes. In this way, we were able to reduce the number of tests conducted and obtain findings for biologically related genes. When 270 up-regulated genes in AGS by *Hp* co-culture were classified according to GO, 13 meaningful categories covered 90 up-regulated genes, and were categorized as genes with the following properties; endopeptidase activity, receptor binding, integrin binding, inorganic phosphate transporter activity, DNA binding, growth factor, caspase, and cytokine activity. These results suggest

that *H. pylori* adhesion to AGS cells trigger the expressions of the following; enzymes, binding or signal transport proteins, apoptotic proteins, and proteins involved in inflammatory reactions. Most of these are known to be involved in the pathological process of *H. pylori*-induced gastroduodenal diseases. In addition, these proteins were closely related to each other. That is, *H. pylori*-stimulates IL8 and promotes cell proliferation though transactivation of epidermal growth factor receptor (EGFR) by ADAM (a disintegrin and metalloproteinase),[14] which is in the endopeptidase activity GO category. ADAM has been found to be strongly expressed after bacterial-epithelial interactions in *H. pylori* positive mucosa.[8,15] USP7, one of ubiquitin-specific processing proteases (USP3, USP7, USP18, USP32), also known as herpesvirus-associated ubiquitin-specific protease (HAUSP), specifically deubiquitylates and stabilize p53 to promote p53-dependent apoptosis and cell-growth arrest. It is interesting that cytokine activity genes such as IL1B, IL8, and TNF showed very high up-regulation up to 60.1-fold in the case of IL1B. This finding suggests that immune mechanism-related genes are markedly affected by *H. pylori* infection, thus aiding colonization and *H. pylori* adaptive acclimatization in the host stomach.

In contrast, for down-regulated genes, the significance of GO category was weak. However, all 5 selected genes (ARF3, M17S2, DDB2, AWP1, and WTAP) were confirmed by real-time RT-PCR, suggesting that the discovery of 20 down-regulated genes would be meaningful. Significant GO categories were GTP, DNA, and protein binding. Of these 20 genes down-regulated in *Hp*/AGS co-culture TRAF4 (TNF receptor-associated factor 4), in the DNA binding GO category, appears meaningful due to the following reasons. Toll-like receptors (TLR) and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase play an essential role in intracellular eradication of engulfed pathogens, and has been found that TRAF4 participates in the molecular mechanism underlying the silencing of TLR-mediated signaling by interacting with molecules harboring phagosome/endosome membranes.[16] Thus *H. pylori* appears to cleverly down regulate TRAF4 in host cells, and thus invades the host cells more easily. Similarly, FAF1 [Fas (TNFRSF6) associated factor 1], which is in the protein binding GO category, was found to be involved in the negative regulation of NF-kappaB activation induced by tumor necrosis factor (TNF)-alpha, interleukin-1beta, and lipopolysaccharide.[17] In addition, FAF1 serves as a scaffolding protein that regulates protein degradation in the ubiquitin-proteasome pathway,[18] and prevents cells from recovery after stress by binding and inhibiting the chaperone activity of Hsp70.[19] Taken together, *H. pylori* appears to have another control system for the activation of NF-kappaB activation and Hsp70 by down-regulation of FAF1. Furthermore, FAP1 has been found to be down-regulated in gastric carcinoma,[20] most of which is caused by *H. pylori* infection.

In conclusion, using oligonucleotide microarray, a total of 270 and 19 genes of AGS cells were found to be up- or down-regulated, respectively, by *H. pylori* adhesion. In addition, function of these genes were classified depending on GO analysis. Furthermore, 16 up- or 5 down-regulated genes among 20 and 5 genes, which have been tried to be confirmed by AOD assay, were confirmed by real-time RT-PCR. These results suggest that *H. pylori* adhesion affect host cell gene expressions which are especially related with endopeptidase activity, receptor or DNA binding, cytokine activity, and caspase activity. These processes contribute to the pathogenic mechanism of *H. pylori*-induced gastroduodenal disease.

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