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The effect of rebamipide on Helicobacter pylori *extract-mediated changes of gene expression in gastric epithelial cells*

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SUMMARY

Background: Recent studies have shown that *Helicobacter pylori* affects intracellular signal transduction in host cells, leading to the activation of transcriptional factors and the induction of pro-inflammatory cytokines. On the other hand, rebamipide, an anti-gastritis and anti-ulcer agent, could scavenge reactive oxygen species and reduce interleukin-8 (IL-8) expression in gastric epithelial cells induced by *H. pylori*-stimulation through the attenuated activation of nuclear factor-κB (NF-κB).

Aims: In this study, we investigated the effects of rebamipide on gene expression in *H. pylori*-stimulated epithelial cells using DNA chip.

Methods: *H. pylori* water extract (HPE) was prepared from NCTC11637, the type strain of *H. pylori*. Total RNA was extracted from MKN45 cells, a human gastric cancer cell line, following HPE-stimulation with and without rebamipide for 3 h, and differences in gene expression profiles were observed using GeneChip and Human 6800 probe array.

Results: The GeneChip analysis demonstrated that 132 up-regulated genes and 873 down-regulated genes, such as growth factors, chemokines and transcription factors, were detected in MKN45 cells 3 h after stimulation of *H. pylori*. Among them, several genes, including bFGF, RANTES and MIP-2β, were previously unknown to be expressed in *H. pylori*-stimulated human gastric cells. Rebamipide reduced expression of 119 genes encoding cytokines, growth factors and their receptors and transcription factors.

Conclusions: These findings suggest that rebamipide could inhibit inflammatory reactions and tumour progression by modifying *H. pylori* infection-induced gene expression in gastric epithelial cells.

INTRODUCTION

Helicobacter pylori is regarded as an important pathogen in patients with active chronic gastritis and as a major risk factor for the development of peptic ulcer and gastric cancer.[1–3] Because peptic ulcer disease and gastric cancer only occur in a subset of individuals with chronic *H. pylori* infection, both bacterial and host factors are presumed to contribute to this differential response. Regarding the influence of *H. pylori* on the host, recent studies have shown that this organism affects intracellular signal transduction in host cells, leading to the activation of transcriptional factors and the induction of pro-inflammatory cytokines.[4–6]

The recently developed complementary microarray technology allows simultaneous parallel analysis of the expression of hundreds to thousands of genes.[7] *H. pylori* induces complicated changes in the pattern of gene expression by gastric epithelial cells.[8–12] Microarray

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technology is a useful tool for studying these complex phenomena that is likely to provide a better and more rapid understanding of the putative molecular pathways involved in *H. pylori*-related gastric disease.

It was recently reported that rebamipide, an anti-gastritis and anti-ulcer agent, alters the activity of nuclear factor-κB (NF-κB) and suppresses interleukin-8 expression in gastric epithelial cells.[13] NF-κB is known to be involved in controlling the cytokine-induced expression of many immune response and inflammatory response genes.[14] Therefore, rebamipide seems likely to also affect the expression of other genes in *H. pylori*-infected tissues.

In this study, we examined the mRNA profile of human gastric epithelial cells incubated with or without *H. pylori* water extract (HPE) by using the DNA microarray technique. It has been reported that HPE has many kinds of pro-inflammatory activity: up-regulation of adhesion molecules on neutrophils[15] and endothelial cells[16]; superoxide production from neutrophils;[17] inhibition of duodenal mucosal alkaline secretion;[18] gastrin release from isolated G cells;[19] IL-8 production from gastric epithelial cells[20] and macrophages[21]; and apoptosis of endothelial cells.[22] These data suggest that HPE may be involved in signal transduction and gene expression associated with inflammatory reactions. In addition, we also assessed the effect of rebamipide on the changes of gene expression due to HPE stimulation by using the same method.

MATERIALS AND METHODS

Helicobacter pylori *water extract (HPE)*

HPE was prepared from NCTC11637, the type strain of *H. pylori,* positive for CagA and VacA. The organism was grown on blood agar plates as previously described.[15] The growth medium consisted of Bacto brain-heart infusion, with 0.5% Bacto (Difco, Detroit, MI, USA) yeast extract, 2.0% Bacto agar, and 7% fresh horse blood. Plates were inoculated and the bacteria were grown for 12 passages in a CO_2 incubator with 12% CO_2 and 100% humidity for 48 h. Cells were harvested with sterile cotton swabs and suspended in distilled water using 1.0 mL per plate (10^9–10^{10} bacteria). The cell suspension was kept at room temperature for 20 min before centrifugation at 17 000 *g* for 15 min. The resulting supernatant, the initial water extract with no preservatives added, was stored at − 20 °C until needed. Before use, the extract was brought to room temperature and centrifuged at 38 700 *g* for 20 min, and the pellet was discarded. The supernatant then was passed through a 0.2 micron Acrodisc (Gelman Science, Ann Arbor, MI, USA) syringe-adapted filter. This procedure removes much of the high molecular weight complex material, which consists mainly of membrane vesicles and intact flagellae.

Treatment of MKN45 cells with HPE

MKN45,[23] a cell line established from a poorly differentiated gastric adenocarcinoma, was cultured in 10-cm culture dishes in RPMI 1640 medium containing 10% fetal bovine serum and antibiotics with or without HPE (10%). After 3 h, 5×10^7 MKN45 cells were collected, washed three times with phosphate-buffered saline, and lysed in ISOGEN reagent (Nippon Gene, Tokyo, Japan). Expression of interleukin-8 (IL-8) mRNA, which has an important role in the pathogenesis of *H. pylori*-induced gastric mucosal inflammation, has been shown to occur 3 h after HPE stimulation[20] and we wished to examine genetic alternations during the early response to infection, so the time of 3 h was chosen for this study.

Rebamipide treatment of HPE-stimulated MKN45 cells

MKN45 cells were cultured in 10-cm cultured dishes in RPMI 1640 medium containing 10% HPE for 3 h. To evaluate the effects of rebamipide on gene expression in HPE-stimulated MKN45 cells, we added the drug to culture medium with HPE. Since we previously found that the rebamipide concentration in gastric mucosal tissue reached 10 μM to 1 mM after oral administration of a clinically effective dose,[24] we selected a rebamipide concentration of 100 μM for this study. After 3 h, 5×10^7 MKN45 cells were collected, washed, and lysed in ISOGEN reagent as described above.

Preparation of biotin-labelled complementary RNA and hybridization to DNA chips

Total RNA was extracted from ISOGEN-lysed MKN45 cells by the guanidine thiocyanate-cesium chloride centrifugation method according to the manufacturer's instructions (ISOGEN Reagent; Nippon Gene, Tokyo, Japan). Preparation of cRNA and target hybridization were performed according to the Affymetrix GeneChip

technical manual. Briefly, double-stranded cDNA was prepared from 5 μg of total RNA using Life Technologies Superscript Choice System (Life Technologies, Inc., Gaitherburg, MD, USA) and an oligo-(dT)24 anchored T7 primer. Biotinylated RNA was synthesized from the double-stranded cDNA by *in vitro* transcription using an Enzo BioArray High Yield RNA Transcript Labeling Kit (Enzo Diagnostic, Inc., Farmingdale, NY, USA). Transcription products were purified using a Qiagen RNA-easy column (Qiagen Inc., Valencia, CA, USA). After biotinylation, the *in vitro* transcription products were fragmented for 35 min at 94 °C in a buffer composed of 200 mM Tris acetate (pH 8.1), 500 mM potassium acetate, and 150 mM magnesium acetate. Then Affymetrix GeneChip arrays (Hum6800 array) were hybridized with the biotinylated products (10 μg/chip) for 16 h at 40 °C using the manufacturer's hybridization buffer. After washing the arrays, hybridized RNA was detected by staining with streptavidin-phycoerythrin (6 × SSPE, 0.05% Triton X-100, pH 7.6, 1 mg/mL acetylated bovine serum albumin, and 2 μg/mL of streptavidin-phycoerythrin from Molecular Probes) for 10 min at 40 °C. The DNA chips were scanned using a specially designed confocal scanner made for Affymetrix by Hewlett-Packard (available through Affymetrix, Santa Clara, CA, USA). Digitized image data were processed using GeneChip software (version 3.0) from Affymetrix, as described previously.[25] The Hum6800 array included several housekeeping genes, such as human beta-actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), to serve as internal controls. The amount of RNA was determined based on the average of the differences between perfect match and mismatch intensities for each probe family. As replicate assays were not performed, a very stringent cut-off point was selected for detecting significant up-regulation or down-regulation of genes, i.e. a 2.5-fold difference in RNA amount between the arrays.

Analysis of mRNA expression by RT-PCR

RT-PCR analysis was used to verify the DNA chip data. The complementary DNA (cDNA) templates for RT-PCR were synthesized from 2 μg of total RNA using 100 U/mL of reverse-transcriptase (Takara Biomedicals, Shiga) and 0.1 μM of oligo (dT)-adapter primer (Takara Biomedicals) in a 50-μL reaction mixture. Five microlitres of cDNA from the sample were amplified with 0.2 μM of the sense and antisense primers for the target genes in a 50-μL reaction mixture containing 75 U/mL of Takara Taq (Takara Biomedicals). After an initial denaturation at 96 °C for 5 min, various cycles of denaturation (96 °C for 20 s), annealing (58 °C for 30 s), and extension (72 °C for 1 min) were performed on a Takara Thermal Cycler MP (Takara Biomedicals). The specific primers[26–28] used for PCR are shown in Table 1. Following PCR, 10 μL of the total amplified product was electrophoresed on ethidium bromide-stained 0.9% agarose gels and visualized under UV fluorescence. Densitometric analysis of PCR-amplified bands was performed with NIH Image Software. Each gel image was imported into NIH Image with Photo-Shop (Adobe systems, CA, USA), a gel-plotting macros was used to outline the bands, and the intensity was calculated on the uncalibrated optical density setting. The relative expression levels were calculated as the density of the product of the respective target genes divided by that of GAPDH from the same cDNA.

RESULTS

Gene expression profile of MKN45 cells with and without HPE stimulation

Expression of 132 genes showed at least 2.5-fold up-regulation after 3 h of HPE stimulation when compared with nonstimulated cells and 873 genes

Table 1. Primer sequences for RT-PCRs

Gene		Primer sequence	Product size (bp)
bFGF[26]	sense	5′-CTGTACTGCAAAAACGGG-3′	349
	antisense	5′-AAAGTATAGCTTTCTGCC-3′	
RANTES[27]	sense	5′-TGCCTCCCATATTCCTCGG-3′	211
	antisense	5′-CTAGCTCATCTCCAAAGA-3′	
MIP-2β[28]	sense	5′-GCTTCCCGACGCGTCTGCTGA-3′	417
	antisense	5′-GTAAGGGCAGGGACCACCCTG-3′	
GAPDH	sense	5′-ACCACAGTCCATGCCATCAC-3′	452
	antisense	5′-TCCACCACCCTGTTGCTGTA-3′	

showed at least 2.5-fold down-regulation after 3 h of HPE stimulation. Only the genes demonstrating greater than 2.5-fold alterations and related to cytokines, cell growth, apoptosis, cell cycle, cell interaction, signal transduction, transcription factor, stress response oncogenesis, and tumour suppression are listed in this report (Tables 2 and 3). While several of the genes have already been reported in other studies, numerous additional genes were shown to be affected by HPE stimulation in this study. The HPE-stimulated MKN45 cells showed up-regulation of mRNA for some cytokines and chemokines, such as interferon-alpha, macrophage inflammatory protein 2-beta (MIP-2β), chemokine exodus, and melanoma-growth stimulating factor (GRO alpha) (Table 2). There was also up-regulation of some of the genes related to cell proliferation, such as cyclin A1, Fas ligand, MAP kinase-activated protein kinase, basic fibroblast growth factor (bFGF), and bFGF receptor (Table 2). Moreover transcription-related genes were up-regulated, including nuclear factor κ-B2 homolog (Table 2). On the other hand, some of the stress-related genes were down-regulated in HPE-stimulated MKN45 cells, including heat shock protein, heat shock factors 1 and 2, and metallothionein-le (Table 3).

Gene expression profile of HPE-stimulated MKN45 cells treated with rebamipide

Fifty genes showed at least 2.5-fold up-regulation of expression and 119 genes showed at least 2.5-fold down-regulation in HPE-stimulated MKN45 cells treated with rebamipide when compared with cells that were incubated with HPE alone. Only those genes demonstrating greater than 2.5-fold alteration and related to inflammation or carcinogenesis are listed (Tables 4 and 5). Among those genes, some cytokine- and chemokine-related genes, such as RANTES, interleukin-8 receptor and interferon-alpha receptor were down-regulated in rebamipide-treated cells. Some growth factor genes, such as insulin-like growth factor 2 and bFGF, were also down-regulated.

Semiquantitave RT-PCR analysis

To confirm the DNA chip data, semiquantitative RT-PCR was performed for some of the altered genes which were not previously known to be expressed in *H. pylori*-stimulated human gastric cells. bFGF mRNA expression was increased in HPE-stimulated MKN45 cells when compared with unstimulated cells. Treatment with rebamipide caused a decrease in the overexpression of bFGF mRNA induced by HPE. A remarkable increase in the RANTES mRNA level was observed in HPE-stimulated MKN45 cells compared with unstimulated cells and rebamipide attenuated its overexpression induced by HPE. Moreover, MIP-2β mRNA levels were increased in HPE-stimulated MKN45 cells when compared with unstimulated cells. Rebamipide did not affect the overexpression of MIP-2β induced by HPE (Figure 1).

DISCUSSION

The DNA microarray is a powerful and sensitive technique that permits the simultaneous screening of tens of thousands of genes and it has been used in both basic and clinical research. It has been suggested that *H. pylori* induces complex changes of gene expression in infected tissues and that these changes eventually result in many diseases such as chronic gastritis, peptic ulcer, and malignancies (including gastric adenocarcinoma and MALT-lymphoma).[1–3, 29] Microarray methods, the including cDNA microarray and the DNA chip, are useful for studying gene expression profile, such as the effects of *H. pylori* infection on epithelial cells in the stomach. In this study, we applied the DNA chip technique to examine effects of rebamipide on these gene expressions as well as the gene expression profile in MKN 45 cells stimulated with HPE.

Several studies have recently assessed the gene expression profile in *H. pylori*-stimulated gastric epithelial cells using the microarray method.[8–12] Although a large number of genes were affected by co-culture of cells with *H. pylori*, the results of these studies were not identical with each other. The different findings may have resulted from differences of many factors such as the cell line, the strain of *H. pylori*, and the microarray used in each experiment, or the time for which gastric epithelial cells were incubated with *H. pylori*. In each experiment, several surprising genes were shown to be affected by *H. pylori* infection. Chiou *et al.*[8] reported that expression of 38 genes was changed after co-incubation of AGS cells with *H. pylori*. Among them, transcription factors (such as c-jun, BTEB-2, and ETR101) and genes involved in signal transduction pathways (such as MAP kinase, interleukin-5, and insulin-like growth factor) were overexpressed. Cell cycle regulatory genes (such as CDC25B and p55 CDC) and stress-induced genes

Table 2. Selected genes up-regulated by HPE-stimulation of MKN45 cells

Function	Gene ID	Gene or encoded protein	Fold difference
cytokine related	M57731	gro-beta mRNA	5.67
cytokine related	X54489	melanoma growth stimulatory activity (MGSA)	5.54
cytokine related	U11877	interleukin-8 receptor type B (IL8RB)	> 10.00
cytokine related	J03171	interferon-alpha receptor (HuIFN-alpha-Rec)	> 10.00
cytokine related	X66365	PLSTIRE for serine/threonine protein kinase (cdc-2 related protein kinase)	> 10.00
cytokine related	M21121	T-cell-specific protein (RANTES) mRNA	> 10.00
cytokine related	X0291	tumour necrosis factor (TNF-alpha)	> 10.00
cytokine related	M27492	interleukin 1 receptor mRNA	> 10.00
cytokine related	U11878	interleukin-8 receptor type B (IL8RB) mRNA	> 10.00
cytokine related	U70136	megakaryocyte stimulating factor mRNA	> 10.00
cytokine related	U64197	chemokine exodus mRNA	> 10.00
cytokine related	X01057	interleukin-2 receptor	> 10.00
cytokine related	M23178	Human homologue-1 of gene encoding alpha subunit of murine cytokine(MIP1/SCI)	> 10.00
cytokine related	M65290	natural killer cell stimulatory factor (NKSF) mRNA	> 10.00
cytokine related	M59465	tumour necrosis factor alpha inducible protein A20 mRNA	> 10.00
cytokine related	M27318	interferon (IFN-alpha-M1) mRNA	> 10.00
cytokine related	M28130	interleukin 8 (IL8) gene	> 10.00
cytokine related	U32659	IL-17 mRNA	> 10.00
cytokine related	X53800	macrophage inflammatory protein-2beta (MIP2beta)	> 10.00
cytokine related	Y00787	MDNCF (monocyte-derived neutrophil chemotactic factor)	> 10.00
cytokine related	X04602	interleukin BSF-2 (B-cell differentiation factor)	> 10.00
cytokine related	M87507	interleukin-1 beta convertase (IL1BCE) mRNA	2.28
cytokine related	X53296	IRAP	2.59
cytokine related	U19713	allograft-inflammatory factor-1 mRNA	3.72
cell growth		Fibroblast Growth Factor Receptor K-Sam	> 10.00
cell growth	M27968	basic fibroblast growth factor (FGF) mRNA	> 10.00
cell growth	X79066	ERF-1 mRNA	> 10.00
cell growth	U40705	Homo sapiens telomeric repeat binding factor (TRF1) mRNA	> 10.00
apoptosis	U86214	Fas-associated death domain protein interleukin-1b-converting enzyme 2 mRNA	6.61
apoptosis	X75346	MAP kinase activated protein kinase	5.51
apoptosis	U82987	Bcl-2 binding component 3 (bbc3) mRNA	3.19
apoptosis	U63295	Human seven in absentia homolog mRNA	4.13
apoptosis	D83699	brain 3 UTR of mRNA for neuronal death protein	> 10.00
apoptosis	S82185	BRAG-1 = brain-related apoptosis gene/Bcl-2 homolog	> 10.00
apoptosis	U45880	X-linked inhibitor of apotosis protein XIAP mRNA	> 10.00
apoptosis	U37546	IAP homolog C (MIHC) mRNA	> 10.00
cell cycle	U66838	Human cyclin A1 mRNA	> 10.00
cell cycle	U10991	Human G2 protein mRNA	> 10.00
cell cycle	D85423	Cdc5	> 10.00
cell cycle	M74091	cyclin mRNA	> 10.00
cell cycle	X66358	KKIALRE for serine/threonine protein kinase	> 10.00
cell cycle	X66360	PCTAIRE-2 for serine/threonine protein kinase	> 10.00
cell cycle	Z22780	*H. sapiens* cylicin mRNA	> 10.00
cell interaction	U56102	adhesion molecule DNAM-1 mRNA	5.70
cell interaction	J03925	Mac-1 gene encoding complement receptor type 3, CD11b	2.65
cell interaction	L11372	protocadherin 43 mRNA	> 10.00
cell interaction	Z22865	dermatopontin mRNA	> 10.00
cell interaction	M55024	cell surface glycoprotein P3.58 mRNA	> 10.00
cell interaction	U72661	ninjurin1 mRNA.	> 10.00
cell interaction	X80026	B-cam mRNA	> 10.00
cell interaction	L34059	cadherin-4 mRNA	> 10.00

Table 2. Continued

Function	Gene ID	Gene or encoded protein	Fold difference
cell interaction	AB000895	cadherin FIB1	5.27
cell interaction	M32334	intercellular adhesion molecule 2 (ICAM-2) gene	5.13
cell interaction	S70348	integrin beta 3	2.81
cell interaction	X82693	mRNA for E48 antigen	> 10.00
cell interaction	M31165	tumour necrosis factor-inducible (TSG-6) mRNA fragment, adhesion receptor CD44	> 10.00
oncogenesis	M16750	pim-1	> 10.00
oncogenesis	HG1996-HT2044	Guanine Nucleotide-Binding Protein Rap2	> 10.00
signal transduction	U66464	haematopoietic progenitor kinase (HPK1) mRNA	> 10.00
signal transduction	L27071	tyrosine kinase (TXK) mRNA	> 10.00
signal transduction	L10717	T-cell-specific tyrosine kinase mRNA	> 10.00
transcription factor	S76638	p50-NF-kappa B homolog	5.33
transcription factor	X68505	myocyte-specific enhancer factor 2 (MEF2).	3.83
transcription factor	X90828	transcription factor, Lbx1	3.42
transcription factor	S77154	TINUR = NGFI-B/nur77 beta-type transcription factor homolog	3.27
transcription factor	M57732	hepatic nuclear factor 1 (TCF1) mRNA	2.81
transcription factor	U03494	Transcription Factor Lsf-Id	2.58
transcription factor		Basic Transcription Factor 2, 34 Kda Subunit	2.54
stress response	M93311	metallothionein-III gene	4.56
stress response	X07834	manganese superoxide dismutase (EC 1.15.1.1)	3.62
others	K02215	angiotensinogen mRNA	3.03
others	D38081	thromboxane A2 receptor	> 10.00
others	U14747	Human visinin-like peptide 1 homolog mRNA	> 10.00
others	M18079	intestinal fatty acid binding protein gene	> 10.00
others	M85085	cleavage stimulation factor	> 10.00
others	M74525	HHR6B (yeast RAD 6 homologue) mRNA	2.98
others	D17525	mRNA for precursor of P100 serine protease of Ra-reactive factor	> 10.00
others	S66896	squamous cell carcinoma antigen = serine protease inhibitor	> 10.00
others	M57730	B61	> 10.00
others	X98176	MACH-beta-1 protein	> 10.00
others	U90304	iroquois-class homeodomain protein IRX-2a mRNA	> 10.00
others	X16706	Human fra-2 mRNA	> 10.00
others	M21005	migration inhibitory factor-related protein 8 (MRP8) gene	> 10.00
others	M32879	steroid 11-beta-hydroxylase (CYP11B1) gene	> 10.00
others	X02612	cytochrome $P^1 - 450$	> 10.00
others	M24470	glucose-6-phosphate dehydrogenase	> 10.00
others	S62904	thiopurine methyltransferase	> 10.00
others	M15856	lipoprotein lipase mRNA	> 10.00
others	U49260	mevalonate pyrophosphate decarboxylase (MPD) mRNA	> 10.00
others	M22324	aminopeptidase N/CD13 mRNA encoding aminopeptidase N	> 10.00
others	M23892	15-lipoxygenase mRNA	> 10.00
others	M35531	GDP-L-fucose:beta-D-galactoside 2-alpha-l-fucosyltransferase mRNA	> 10.00
others	M64231	spermidine synthase gene	> 10.00
others	M13928	delta-aminolevulinate dehydratase mRNA	> 10.00
others	D16583	Human gene for L-histidine decarboxylase	> 10.00
others	X77922	GD3 synthase	> 10.00
others	S79862	26 S protease subunit 5b = 50 kDa subunit	> 10.00
others	M29037	17 beta-hydroxysteroid dehydrogenase (17BHSDI) gene	> 10.00
others	L41349	phospholipase C beta 4 (PLCB4) mRNA	> 10.00
others	U24577	LDL-phospholipase A2 mRNA	> 10.00
others	M27783	neutrophil elastase mRNA	> 10.00
others	M13690	plasma protease (C1) inhibitor mRNA	> 10.00
others	L15326	endoperoxide synthase type II mRNA	5.24

Table 2. Continued

Function	Gene ID	Gene or encoded protein	Fold difference
others	U07919	aldehyde dehydrogenase 6 mRNA	4.73
others	U29615	chitotriosidase precursor mRNA	4.12
others	U86409	hyaluronan synthase 3 (HAS3) gene	3.96
others	Y00317	liver microsomal UDP-glucuronosyltransferase (UDPGT)	3.42
others	X56088	cholesterol 7-alpha-hydroxylase	3.42
others	D28235	PTGS2 gene for prostaglandin endoperoxide synthase-2	2.89
others	L11701	phospholipase D mRNA	2.52

Table 3. Selected genes down-regulated by HPE-stimulation of MKN45 cells

Function	Gene ID	Gene or encoded protein	Fold difference
cytokine related	M13207	granulocyte-macrophage colony-stimulating factor (CSF1) gene	< 0.10
cytokine related	V00535	interferon beta 1 gene extracted from Gene for human fibroblast interferon beta 1	< 0.10
cytokine related	X81851	IL-4 gene splice variant	< 0.10
cytokine related	M28983	interleukin 1 alpha (IL 1) mRNA	< 0.10
cytokine related	U83326	CC chemokine receptor-5 (CCR5) gene	< 0.10
cytokine related	U83303	GCP-2 gene (granulocyte chemotactic protein-2)	< 0.10
cytokine related	M31166	tumour necrosis factor-inducible (TSG-14) mRNA	0.13
cytokine related	U31628	interleukin-15 receptor alpha chain precursor (IL15RA) mRNA	0.14
cytokine related	X58298	interleukin-6-receptor	0.14
cytokine related	X04500	prointerleukin 1 beta	0.16
cytokine related	M58286	tumour necrosis factor receptor mRNA	0.18
cytokine related	U32324	interleukin-11 receptor alpha chain mRNA	0.21
cytokine related	M20137	interleukin 3 (IL-3) mRNA	0.21
cytokine related	U31120	interleukin-13 (IL-13) precursor gene	0.22
cytokine related	X00695	interleukin-2 (IL-2) gene	0.22
cytokine related	U14407	interleukin 15 (IL15) mRNA	0.24
cytokine related	U00672	interleukin-10 receptor mRNA	0.27
cytokine related	U69108	TNF receptor associated factor 5 mRNA	0.29
cytokine related	X17648	granulocyte-macrophage colony-stimulating factor receptor (hGM-CSF-R)	0.30
cytokine related	M69203	cytokine (SCYA2) gene	0.32
cytokine related	K03515	neuroleukin mRNA, complete cds	0.32
cytokine related	U52682	lymphocyte specific interferon regulatory factor/interferon regulatory factor 4 mRNA	0.33
cytokine related	L39064	interleukin 9 receptor (IL9R) gene	0.34
cytokine related	U77396	TNF-alpha inducible responsive element mRNA	0.36
cytokine related	U46767	monocyte chemoattractant protein-4 precursor (MCP-4) mRNA	0.37
cytokine related	Y10659	IL-13Ra mRNA	0.39
cell growth	S72043	GIF = growth inhibitory factor	< 0.10
cell growth	M65062	insulin-like growth factor binding protein 5 (IGFBP-5) mRNA	< 0.10
cell growth	M62302	growth/differentiation factor 1 (GDF-1) mRNA	< 0.10
cell growth	M92934	connective tissue growth factor	< 0.10
cell growth	U03858	flt3 ligand mRNA	< 0.10
cell growth	X16323	hepatocyte growth factor (HGF)	< 0.10
cell growth	M34057	transforming growth factor-beta 1 binding protein mRNA	< 0.10
cell growth	M57399	nerve growth factor (HBNF-1) mRNA	0.11
cell growth	U37055	hepatocyte growth factor-like protein gene	0.15
cell growth	M17863	preproinsulin-like growth factor II (IGF-II) variant mRNA	0.21
cell growth	M34309	epidermal growth factor receptor (HER3) mRNA	0.33
cell growth	L42379	bone-derived growth factor (BPGF-1) mRNA	0.35
cell growth	U41745	PDGF associated protein mRNA	0.37
cell growth	M35878	insulin-like growth factor-binding protein-3 gene	0.40
cell growth		Glial Growth Factor 2	0.40

Table 3. Continued

Function	Gene ID	Gene or encoded protein	Fold difference
cell growth	M38449	transforming growth factor-beta mRNA	0.16
cell growth	L07594	transforming growth factor-beta type III receptor (TGF-beta) mRNA	0.22
cell growth	D50683	TGF-betaIIR alpha	0.33
cell growth	L17075	TGF-b superfamily receptor type I mRNA	< 0.10
cell growth	M77349	transforming growth factor-beta induced gene product (BIGH3) mRNA	0.40
apoptosis	X83492	Fas/Apo-1	0.36
apoptosis	X84213	BCl-2 homologue	0.37
apoptosis	U83598	death domain receptor 3 soluble form (DDR3) mRNA	0.25
apoptosis	U58334	Bcl2, p53 binding protein Bbp/53BP2 (BBP/53BP2) mRNA	0.27
apoptosis	U09477	53BP1 p53-binding protein mRNA	0.32
cell cycle	M17754	BN51	< 0.10
cell cycle		Cyclin D1 Promoter	< 0.10
cell cycle	U53174	cell cycle checkpoint control protein mRNA	0.12
cell cycle	X95406	cyclin E gene.	0.16
cell cycle	X78342	PISSLRE mRNA	0.19
cell cycle	X87843	cyclin H assembly factor	0.28
cell cycle	M81933	cdc25A mRNA	0.30
cell cycle	M74093	cyclin mRNA	0.34
cell cycle	M68520	cdc2-related protein kinase mRNA	0.35
cell cycle	D50310	cyclin I	0.38
cell cycle	X77794	cyclin G1	0.39
cell interaction	AB000897	cadherin FIB3	< 0.10
cell interaction	L34060	cadherin-8 mRNA	0.18
cell interaction	U59289	H-cadherin mRNA	0.26
cell interaction	Y11710	extracellular matrix protein collagen type XIV	0.27
cell interaction	X83228	LI-cadherin	0.29
cell interaction	Z35402	E-cadherin	0.33
oncogenesis		Oncogene Ret/Ptc2	< 0.10
oncogenesis	X57110	c-cbl proto-oncogene	< 0.10
oncogenesis	M98343	amplaxin (EMS1) mRNA	< 0.10
oncogenesis	U33202	mdm2-D (mdm2) mRNA	< 0.10
oncogenesis	M14949	R-ras gene	0.10
oncogenesis	X03072	int-1 mammary oncogene	0.15
oncogenesis	J00277	c-Ha-ras1 proto-oncogene	0.26
oncogenesis	U73737	hMSH6 gene	0.29
oncogenesis	V01512	cellular oncogene c-fos (complete sequence)	0.30
oncogenesis	L00058	(GH) germline c-myc proto-oncogene	0.33
oncogenesis	X03663	c-fms proto-oncogene	0.33
oncogenesis	U77735	pim-2 protooncogene homolog pim-2 h mRNA	0.35
oncogenesis	J04111	c-jun proto oncogene (JUN)	0.38
tumour supressor	X86371	tumour suppressor protein	< 0.10
tumour supressor	D37965	PDGF receptor beta-like tumour suppressor (PRLTS)	< 0.10
tumour supressor	U03056	tumour suppressor (LUCA-1) mRNA	< 0.10
signal transduction	U09607	JAK family protein tyrosine kinase (JAK3) mRNA	< 0.10
signal transduction	L11329	protein tyrosine phosphatase (PAC-1) mRNA	< 0.10
signal transduction	U27193	protein-tyrosine phosphatase mRNA	< 0.10
signal transduction	U06454	AMP-activated protein kinase (hAMPK) mRNA	< 0.10
signal transduction	U43885	Grb2-associated binder-1 mRNA	< 0.10
signal transduction	U67156	mitogen-activated kinase kinase kinase 5 (MAPKKK5) mRNA	< 0.10
signal transduction	X79483	ERK6 mRNA for extracellular signal regulated kinase	< 0.10
signal transduction	U57093	small GTP-binding protein rab27b mRNA	< 0.10
signal transduction	U29725	BMK1 alpha kinase mRNA	< 0.10
signal transduction	U18297	MST1 (MST1) mRNA	< 0.10

Table 3. Continued

Function	Gene ID	Gene or encoded protein	Fold difference
signal transduction	L07868	receptor tyrosine kinase (ERBB4) gene	< 0.10
signal transduction	X61587	rhoG mRNA for GTPase	0.14
signal transduction	L11285	ERK activator kinase (MEK2) mRNA	0.25
signal transduction	M28209	GTP-binding protein (RAB1) mRNA	0.25
signal transduction	X87838	beta-catenin	0.26
signal transduction	M12174	ras-related rho mRNA	0.27
signal transduction	U18671	Stat2 gene	0.28
signal transduction	U59914	Human chromosome 15 Mad homolog Smad6 mRNA	0.33
signal transduction	X60188	ERK1 mRNA for protein serine/threonine kinase	0.34
signal transduction	Z15108	protein kinase C zeta	0.36
signal transduction	U09578	MAPKAP kinase (3pK) mRNA	0.38
signal transduction	U17743	JNK activating kinase (JNKK1) mRNA	0.40
signal transduction	L11695	activin receptor-like kinase (ALK-5) mRNA	< 0.10
transcription factor	S78825	Id1 (Id1-b) = transcription regulator helix-loop-helix protein	< 0.10
transcription factor	X63380	RSRFR2	< 0.10
transcription factor	U80669	androgen regulated homeobox protein (NKX3.1) mRNA	< 0.10
transcription factor	M96980	myelin transcription factor 1 (MTF1) mRNA	< 0.10
transcription factor	M98833	ERGB transcription factor (FLI-1 homolog) mRNA	< 0.10
transcription factor	L39060	transcription factor SL1 mRNA	< 0.10
transcription factor	U75309	TBP-associated factor (hTAFII100) mRNA	1.90
transcription factor	U75308	TBP-associated factor (hTAFII130) mRNA	0.23
transcription factor	U85430	transcription factor NFATx4 mRNA	0.32
transcription factor	L49380	B4 transcription factor ZFM1 mRNA	0.33
transcription factor	X63469	transcription factor TFIIE beta	0.33
transcription factor	U08015	NF-ATc mRNA	0.34
transcription factor	D78261	ICSAT transcription factor mRNA	0.34
transcription factor	U10324	nuclear factor NF90 mRNA, complete cds	0.34
transcription factor	X60787	transcription factor ILF	0.35
transcription factor	X84002	TAFII20 mRNA for transcription factor TFIID	0.36
transcription factor	L41067	NF-AT4c mRNA	0.38
transcription factor	M31523	transcription factor (E2A) mRNA	0.39
transcription factor	M77810	transcription factor GATA-2 (GATA-2) mRNA	< 0.10
transcription factor	M97796	helix-loop-helix protein (Id-2) mRNA	0.14
transcription factor	U08336	basic helix-loop-helix transcription factor mRNA	0.20
transcription factor	L07592	peroxisome proliferator activated receptor mRNA	< 0.10
transcription factor	L02932	peroxisome proliferator activated receptor mRNA	0.27
stress response	U15590	heat shock protein 27 (HSP27) mRNA	< 0.10
stress response	U07807	metallothionein IV (MTIV) gene	< 0.10
stress response	M11717	heat shock protein (hsp 70) gene	< 0.10
stress response	X51757	Human heat-shock protein HSP70B gene	< 0.10
stress response	D85429	heat shock protein 40	< 0.10
stress response	V00594	metallothionein from cadmium-treated cells	0.11
stress response	M10942	metallothionein-Ie gene (hMT-Ie)	0.13
stress response	Z23090	28 kDa heat shock protein	0.15
stress response	L08069	heat shock protein, E. coli DnaJ homologue mRNA	0.18
stress response	J02947	extracellular-superoxide dismutase (SOD3) mRNA	0.22
stress response	M10943	metallothionein-If gene (hMT-If)	0.24
stress response	M96233	glutathione transferase class mu number 4 (GSTM4) gene	0.25
stress response	Y00371	Heat Shock Protein, 70 Kda	0.28
stress response	U40992	heat shock protein hsp40 homolog mRNA	0.28
stress response	L12723	heat shock protein 70 (hsp70) mRNA	0.30
stress response	L26336	heat shock protein HSPA2 gene	0.35
stress response	M65217	heat shock factor 2 (HSF2) mRNA	0.36

Table 3. Continued

Function	Gene ID	Gene or encoded protein	Fold difference
stress response	U22431	hypoxia-inducible factor 1 alpha (HIF-1 alpha) mRNA	0.36
stress response	M64673	heat shock factor 1 (TCF5) mRNA	0.36
stress response	X15183	90-kDa heat-shock protein	0.39
stress response	U39487	xanthine dehydrogenase/oxidase mRNA	0.25
others	U62015	Cyr61 mRNA	0.23
others	M29960	steroid receptor (TR2-11) mRNA	0.19
others	U05255	glycophorin HeP2 mRNA	< 0.10
others	L46720	autotaxin-t (atx-t) gene	< 0.10
others	U16997	orphan receptor ROR gamma mRNA	0.16
others	L06797	Human (clone L5) orphan G protein-coupled receptor mRNA	0.23

Table 4. Selected genes down-regulated by rebamipide administration of HPE-stimulated MKN45 cells

Function	Gene ID	Gene or encoded protein	Fold difference
cytokine related	M27318	interferon (IFN-alpha-M1) mRNA	0.13
cytokine related	J03171	interferon-alpha receptor (HuIFN-alpha-Rec) mRNA	< 0.10
cytokine related	M21121	Human specific protein (RANTES) mRNA	< 0.10
cytokine related	U11878	interleukin-8 receptor type B (IL8RB) mRNA	0.25
cell growth	X58255	Flg-2 gene for fibroblast growth factor receptor	0.38
cell growth	J04513	basic fibroblast growth factor (bFGF) 22.5 kDa, 21 kDa and 18 kDa protein mRNA	0.40
cell growth	HG3543-HT3739	Insulin-Like Growth Factor 2	0.26
cell cycle	M74091	cyclin mRNA	< 0.10
cell interaction	M31165	Human tumour necrosis factor-inducible (TSG-6) mRNA fragment	0.30
cell interaction	L34059	cadherin-4 mRNA	< 0.10
oncogenesis	U08023	cellular proto-oncogene(c-mer)	0.27
cell interaction	L11372	protocadherin 43 mRNA	< 0.10
transcription factor	M57732	hepatic nuclear factor 1 (TCF1) mRNA	< 0.10
transcription factor	X90828	transcription factor, Lbx1	0.33
others	HG3355-HT3580	putative mono-ADP-ribosyltransferase (htMART) mRNA, complete cds	< 0.10
others	M85085	cleavage stimulation factor	< 0.10

(such as heat shock protein 27, heat shock factor protein 1, and glutathione S-transferase T1) were down-regulated. Maeda *et al.*[9] reported that *H. pylori* induced significant changes of eight genes in MKN45 and AGS cells. Among them, IκBα and A20 were overexpressed, which may inhibit *H. pylori*-induced activation of NF-κB, thereby regulating inflammatory and apoptotic responses. Moreover, Sepulveda *et al.*[12] demonstrated that several additional genes, such as pim-1 and ATF3, were affected by *H. pylori* infection to AGS cells using the U95A microarray. In our study, a number of additional genes were shown to be affected after 3 h of stimulation with HPE, and we also demonstrated that the expression patterns of some genes were the same as those previously reported using microarrays. The genes for inflammatory cytokines and chemokines (e.g. interleukin-8, tumour necrosis factor-alpha, interferon-alpha, melanoma growth-stimulatory activity, RANTES, and MIP-2β) and genes for the receptors of these factors (e.g. IFN-α receptor, interleukin-8 receptor, and interleukin-1 receptor) were overexpressed, as well as genes related to cell–cell interactions (e.g. cell surface glycoprotein, ninjurin, and B-CAM), genes related to cell growth (e.g. bFGF, bFGF receptor, and ERF-1), and oncogenes (e.g. pim-1 and Rap2). On the other hand, cell cycle regulatory genes (e.g. BN51, CDC25A, cyclin D1 promoter, and cyclin E), some cell–cell interaction genes (e.g. E-cadherin, extracellular matrix protein

Table 5. Selected genes up-regulated by rebamipide administration of HPE-stimulated MKN45 cells

Function	Gene ID	Gene or encoded protein	Fold difference
cell interaction	M37245	Human Ig superfamily cytotoxic T-lymphocyte-associated protein (CTLA-4) gene	7.95
oncogenesis	M98343	Homo sapiens amplaxin (EMS1) mRNA	9.48
transcription factor	M77698	Homo sapiens GLI-Krupple related protein (YY1) mRNA	14.65
others	M74491	Human ADP-ribosylation factor 3 mRNA	7.00
others	L47738	Homo sapiens inducible protein mRNA, complete cds	5.10
others	L20826	Human I-plastin mRNA, complete cds	4.74
others	HG4660-HT5073	Microtubule-Associated Protein 1b	3.15
others	L11931	Human cytosolic serine hydroxymethyltransferase (SHMT) mRNA	9.00
others	Z36531	H.sapiens mRNA for fibrinogen-like protein (pT49 protein)	2.95
others	U31903	Human CREB-RP (creb-rp) mRNA	2.88

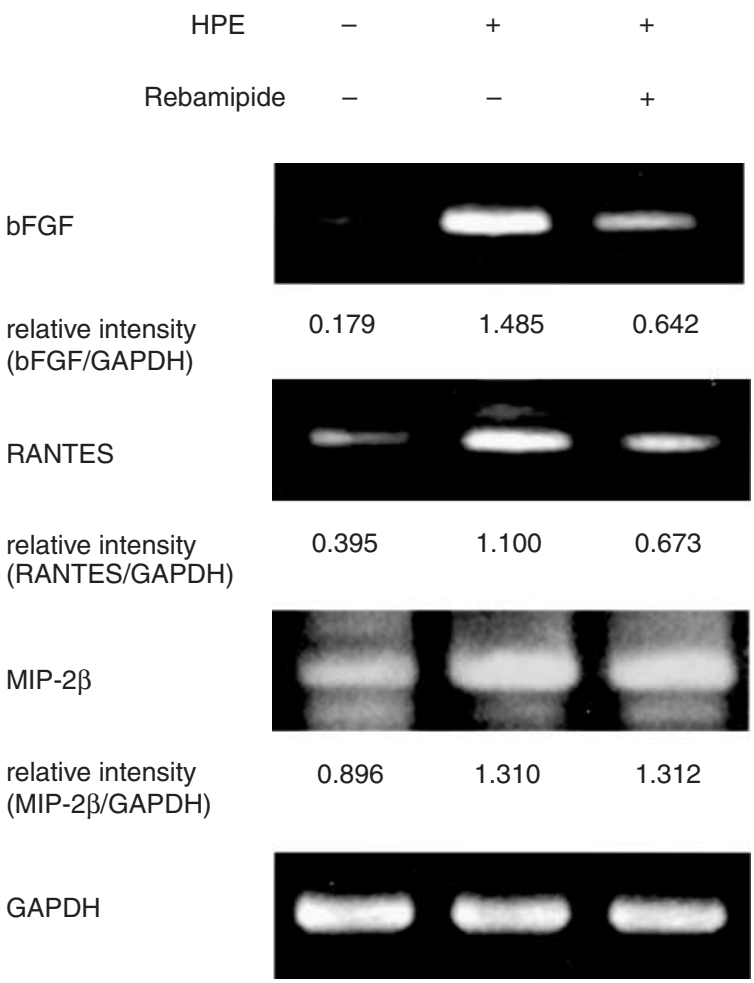

Figure 1. RT-PCR analysis of mRNA expression in HPE-stimulated MKN45 cells with or without rebamipide. Total RNA was extracted from MKN45 following HPE-stimulation with and without rebamipide for 3 h and mRNA expression was analysed by RT-PCR. Three genes (bFGF, RANTES and MIP2β) were tested and GAPDH was used as a control.

collagen type XIV), and stress-related genes (e.g. heat shock proteins 27, 40, and 70, and metallothionein) were down-regulated. Among them were several genes, including bFGF, RANTES, and MIP-2β, which were previously unknown to be expressed in *H. pylori*-stimulated human gastric cells. In these three genes, we analysed mRNA expression by RT-PCR and confirmed mRNA alterations were consistent with the results from DNA chip analysis.

Rebamipide is an anti-ulcer and anti-gastritis agent with several anti-inflammatory actions: it scavenges hydroxyl radicals[24] and inhibits the production of oxygen radicals by activated neutrophils[17, 30, 31]; inhibits neutrophil adhesion to endothelial cells[17]; down-regulates the expression of adhesion molecules[17]; and reduces interleukin-8 production stimulated by *H. pylori* in human gastric cancer cell lines.[32] Suzuki *et al.* also reported that rebamipide could play a role in inhibiting the level of *H. pylori* colonization and gastric lesion formation in Mongolian gerbils.[33] Recently, it was reported that rebamipide inhibited IL-8 expression at least partly through a reduction in the activation of NF-κB and through decreasing the activation of three classes of MAP kinases (JNK/SAPK, ERK1/2, and p38 MAP kinases) induced by *H. pylori.*[13] As expected, rebamipide affected the expression of various genes altered by HPE stimulation in this study. Among these genes, rebamipide inhibited the increased expression of cytokine related genes such as interferon-α, interferon-α receptor, interleukin-8 receptor and RANTES, suggesting that this anti-ulcer drug may be useful for the treatment of gastric inflammation associated with *H. pylori* infection. In addition, rebamipide also reduced expression of several genes (including bFGF, insulin-like growth factor, and cellular proto-oncogene) which might possibly be involved in the progression of *H. pylori*-related diseases.[34, 35] It has been reported that various growth factors, including bFGF, are produced locally by regenerating gastric mucosal cells during the healing of ulcers and that these factors control re-epithelialization and the reconstruction of glandular elements.[35]

We used a gastric cancer cell line stimulated with HPE as a model to study the effects of *H. pylori* infection on gastric epithelial cells. Therefore, we need to take into account the possibility that the gene responses found in this study do not occur in the normal gastric mucosa. Further *in vivo* studies need to be performed to validate the results obtained with the DNA microarray.

Using the microarray technique, we were able to screen a large number of genes in HPE-stimulated gastric epithelial cells and assess the genes that were influenced by rebamipide treatment. These findings may provide an insight into the role of some novel genes in the pathogenetic mechanisms of *H. pylori*. We hope that the gene expression profiles presented here will provide a useful resource as a database for further studies.

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