

Helicobacter pylori infection induced alteration of gene expression in human gastric cells

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Abstract

Background—*Helicobacter pylori*, a human pathogen responsible for many digestive disorders, induces complex changes in patterns of gene expression in infected tissues. cDNA expression arrays provide a useful tool for studying these complex phenomena.

Aim—To identify genes that showed altered expression after *H pylori* infection of human gastric cells compared with uninfected controls.

Methods—The gastric adenocarcinoma cell line AGS was cocultivated with *H pylori*. Growth of infected cells was determined by trypan blue exclusion assay. Complementary DNA probes derived from *H pylori* treated and untreated cells were hybridised to two identical Atlas human cDNA expression arrays, and those genes with altered expression levels were identified. A real time quantitative reverse transcription-polymerase chain reaction assay was used to better define expression patterns of these genes in endoscopically gastric mucosal biopsies with and without *H pylori* infection.

Results—Over 24 hours, cocultivation with *H pylori* inhibited AGS cell growth but did not cause a noticeable degree of cell death. *H pylori* treatment altered the pattern of gene expression in AGS cells. We identified 21 overexpressed genes and 17 suppressed genes from the cDNA expression arrays. The majority of genes were transcription factors such as *c-jun*, *BTEB2*, and *ETR101*. Other genes were involved in signal transduction pathways, such as MAP kinase, interleukin 5, and insulin-like growth factor. Genes involved in cell cycle regulation and differentiation, such as *CDC25B* and *NM23-H2*, were also identified. In patients with *H pylori* infection (n=20), there was a significant difference for *ERCC3*, *Id-2*, and *NM23-H2* mRNA levels in infected gastric mucosa compared with uninfected gastric mucosa in patients without peptic diseases (n=20) (*ERCC3* 4.75 molecules/10⁴ β-actin mRNA molecules *v* 13.65, *p*<0.001; *Id-2* 16.1 *v* 23.4, *p*<0.05; *NM23-H2* 17.5 *v* 45.5, *p*<0.001). There was no significant difference between mRNA levels of *c-jun* and *CDC25B* in *H pylori* colonised gastric mucosa and uninfected mucosa.

Conclusion—We demonstrated that *H pylori* infection caused alteration of gene expression in AGS cells. The differential hybridisation technique of Atlas human cDNA expression array is a useful method

to identify host genes involved in pathogenic mechanisms in *H pylori* infection.

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Keywords: gastric adenocarcinoma; *Helicobacter pylori*; cDNA microarray; gene expression; transcription factors; signal transduction pathway

Helicobacter pylori, a human pathogen, infects gastric mucosa and causes many digestive disorders such as peptic ulcers, chronic gastritis, and gastric cancer.¹ The virulence factors of *H pylori* include those allowing the bacterium to survive in the hostile environment of the gastric lumen, such as spiral shape and motility, adaptive enzymes and proteins, and ability to adhere to gastric mucosal cells and mucus.^{2,3} Additional factors considered as pathogenic mechanisms of *H pylori* include those causing disruption of the gastric mucosal barrier, such as vacuolating toxin (VacA), mediators of inflammation, and contributors to gastric acid.^{4–6} However, peptic ulcer diseases and gastric cancer occur in only a subset of individuals chronically infected with *H pylori*. Therefore, both bacterial and host factors are presumed to contribute to this differential response. Regarding the host cellular response, in vivo and in vitro studies have shown that *H pylori* infection causes inflammation of gastric epithelium cells by increasing interleukin 8 (IL-8) expression and secretion.^{7–9} *H pylori* infection also increases pepsinogen secretion and may result in damage of epithelium integrity.^{10–12} Recent studies reported that *H pylori* caused apoptosis of infected epithelium cells.^{13–15} Moreover, they produce oxidative stress in gastric mucosa.^{16,17}

Investigating gene expression of host cells during pathological changes that arise in diseases such as peptic ulcers and even cancer may provide the information we need to analyse the biological processes induced by *H pylori* infection. In a previous study, we demonstrated that *H pylori* induced alteration of gene expression in human gastric cells using the technique of mRNA differential display.¹⁸ cDNA expression array is a new technology which offers high throughput screening of the differential expression of many genes in pathogenic cells.¹⁹ This technology is based on the principle of reverse northern hybridisation.²⁰ Hundreds to tens of thousands of DNA fragments are fixed on support material such as nylon membranes or glass slides. Genes with different expression levels can be identified by

Abbreviations used in this paper: VacA, vacuolating toxin; IL, interleukin; FBS, fetal bovine serum; RT-PCR, reverse transcription-polymerase chain reaction; IL-5R, interleukin 5 receptor; IGF2, insulin-like growth factor binding protein 2.

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comparing two or more duplicate arrays hybridised with cDNA probes derived from different samples. Until now, cDNA expression arrays have been used to study differentially expressed genes in several types of cancers.²¹⁻²⁴ The technology also has potential for studying other cellular behaviours, such as differentiation and drug response.^{25 26}

In this study, we used a cDNA expression array assay to analyse alterations in gene expression in cultured gastric epithelium cells after *H pylori* infection. Of the altered genes, only a small proportion have previously been identified, such as those for cytokine expression, apoptosis, and signal transduction. Many other genes have not been studied in relation to *H pylori* induced responses. In this study, the possible functions of these genes and their relationship to disease is discussed.

Patients and methods

PATIENTS

Gastric biopsies were obtained from 20 *H pylori* negative patients (10 men and 10 women) and 20 *H pylori* positive patients, including 16 with duodenal ulcer (10 men and six women) and four with gastric ulcer (two men and two women). Biopsies were frozen in liquid nitrogen until use. Diagnosis was made based on endoscopic examination, histological examination of gastric biopsy, and the rapid urease (CLO) test.

BACTERIUM AND CELL LINE CULTIVATION

The *H pylori* strain K8, positive for VacA and CagA, was isolated from an ulcer patient at Chang Gung Memorial Hospital, Taiwan. The bacterium was cultured on chocolate agar plates at 37°C in a microaerophilic atmosphere of 10% CO₂ in air and 95% humidity. The human gastric adenocarcinoma cell line AGS (CCRC 60102) was obtained from the Food Industry Research and Development Institute, Taiwan. AGS cells were cultured in Ham's F12 medium (GibcoBRL, Grand Island, New York, USA) containing 10% fetal bovine serum (FBS) (Trace Bioscience, Australia) and grown in an incubator at 37°C with 5% CO₂ in air.

COCULTIVATION OF H PYLORI AND AGS CELLS

Prior to the experiment, *H pylori* were scraped from agar plates, washed once with phosphate buffer saline, and resuspended in serum free F12 medium. The concentration of bacterium was adjusted to 1×10⁸ cells/ml, as measured under a microscope on a haemocytometer and by spectrophotometry at 625 nm. AGS cells were seeded on 100 mm dishes 18 hours before bacterial cells were added. To determine cell growth rate, 5×10⁵ AGS cells were treated with 1×10⁸ or 2×10⁸ bacterial cells in 5 ml of medium without antibiotics. Cells were trypsinised after 24 and 96 hours, and cell numbers and their viability were measured on a haemocytometer by trypan blue exclusion. For RNA extraction, 2×10⁶ AGS cells were incubated with 2×10⁸ bacteria in 10 ml of medium. Cells treated with an equal volume of bacterium free medium served as controls. After 24

hours, 2×10⁷ cells were collected, washed three times with phosphate buffer saline, and lysed in TRIzol reagent (GibcoBRL).

EFFECT OF H PYLORI ON AGS CELL APOPTOSIS

Synchronised AGS cells cocultured with *H pylori* were analysed in parallel with cells grown in the absence of microorganisms to determine effects on cell apoptosis. Synchronised AGS cells were serum deprived for 48 hours. Adherent and floating cells were collected together and fixed in 70% ethanol. Cell pellets were suspended in 400 µl of 0.2 mg/ml propidium iodide containing 0.6% NP40 (ICN Pharmaceuticals, Costa Mesa, California, USA) with the same volume of 2 mg/ml RNase (Sigma Chemical Co, St. Louis, Missouri, USA) and incubated in the dark at room temperature for 30 minutes. Cell suspensions were filtered through a 60 µm mesh filter. Data acquisition and analysis were performed on a FACScan instrument (Becton Dickinson, San Jose, California, USA). Apoptotic cells were considered to constitute the sub-G₁ cell population. All experiments were performed at least three times.

COMPLEMENTARY DNA PROBE PREPARATION AND HYBRIDISATION OF HUMAN DNA EXPRESSION ARRAY

To the TRIzol lysed cells, a 0.2 volume of chloroform was added and the aqueous phase containing RNA was collected after centrifugation. RNA was precipitated in 50% isopropanol and the pellets were washed with 70% alcohol, dried, and resuspended in diethylpyrocarbonate treated H₂O. Poly A+ mRNA was isolated from 500 µg of total RNA using oligotex mRNA kits (Qiagen, Hilden, Germany). cDNA probe preparation and membrane hybridisation were performed according to the manufacturer's instructions for Atalas human cDNA expression array (Clontech, Palo Alto, California, USA). Briefly, 1 µg of mRNA was reverse transcribed into cDNA by MMLV reverse transcriptase in the presence of CDS primer mix and α³²P-dATP (3000 Ci/mmol; Amersham Pharmacia, Hong Kong). Labelled cDNA was separated from unincorporated nucleotides using a CHROMA SPIN-200 column (Clontech). The human cDNA expression arrays were prehybridised at 68°C for 30 minutes in ExpressHyb solution (Clontech) to which 0.1 mg/ml of salmon sperm DNA (GibcoBRL) had been added. cDNA probes were hybridised to the arrays at 68°C overnight. Membranes were washed four times with solution 1 (2× SSC and 1% SDS) and twice with solution 2 (0.1× SSC and 0.5% SDS) for 30 minutes at 68°C in all cases and exposed to a phosphor screen. The images and quantitative data of gene expression levels were analysed using PhosphoImager (Molecular Dynamics, Sunnyvale, California, USA).

NORTHERN BLOTTING

For northern blots, total RNA (10 µg) obtained from untreated AGS and *H pylori* treated AGS cells were separated on formaldehyde denaturing 1% agarose gels and transferred to nylon

membranes (Schleicher and Schuell, Hahnstrasse, Germany). After UV cross linking, blots were prehybridised at 40°C in 1× SSC, 1% SDS, 1× Denhardt's solution, 200 µg/ml of salmon sperm DNA, and 20 mM Tris buffer (pH 7.1) for four hours. Hybridisation was continued at 40°C overnight in the presence of 1×10⁶ cpm/ml of random prime ³²P labelled probe. After washing at room temperature in 2× SSC/0.1% SDS, blots were washed at 65°C for 20 minutes with 1× SSC/0.1% SDS. The washed membrane was autoradiographed for six hours. Temporal changes in expression of genes in *H pylori* treated and untreated AGS cell lines were studied by northern hybridisation analysis. For the loading control, blots were stripped and reprobated with a ³²P labelled human β-actin gene probe. The probes for the genes were made by reverse transcription-polymerase chain reaction (RT-PCR) using specific primer sets. Complementary DNA was synthesised from 2 µg of total RNA in a 25 µl reaction mixture containing 1× reverse transcriptase reaction buffer (Promega, Madison, Wisconsin, USA), 200 µM of dNTPs, 10 ng of oligo (dT)₁₅ primer, 8 mM dithiothreitol, 40 U of Rnasin (Promega), and 100 U of M-MLV reverse transcriptase (Promega). The mixture was incubated at 37° C for 50 minutes, heated to 80°C for 10 minutes, and chilled on ice. Amplification of each specific gene was performed using a 2 µl aliquot of cDNA in a 50 µl amplification mixture containing 200 mM dNTPs, 0.2 µM forward and reverse primers, 2.5 U of Taq DNA polymerase, and 1× Taq reaction buffer. For β-actin, PCR amplification was performed for 22 cycles (94°C for 30 seconds, 50° C for 30 seconds, 72°C for 30 seconds) after a first denaturing step (94°C for two minutes). For the other genes, PCR amplification was performed for 25–30 cycles (94°C for 30 seconds, 60° C for 30 seconds, 72°C for 30 seconds). The specific primers used for PCR were as follows: 5'-CCGAAGGCTA GTGCGATGTTTC-3' and 5'-ACTTGATG CAATCCAAACTTTGAA-3' for *c-jun*; 5'-AGATTTACCCTGTGGATCTGTCACACT-3' and 5'-TCATCACAGAGACTTTTCAC AAG CAA-3' for *EERC3*; 5'-ATATCAGCATCC TGTCCTTGCA-3' and 5'-GAAA TCA TG AACACCGCTTATTCA-3' for *Id-2*; 5'-CTG AACCTTGTGGATTGGAGTTC-3' and 5'-AAAAGCTGCAGGATGTCACCA-3' for *p55 CDC*; 5'-CAGTAGACGGAAAGCACCAAG A-3' and 5'-AATCACAAACTTATCCACGAT GTTG-3' for *CDC25B*; 5'-ACCTGAAAGA CCGACCATTCTTCC-3' and 5'-AGACTGC TGTTGTGTCCACCTC-3' for *NM23-H2*; and AAGATGACCCAGATCATGTT-3' and GCGACATAGCACAGCTTCT-3' for β-actin. After amplification, PCR products were separated on a 2.5% agarose gel and stained with ethidium bromide.

QUANTITATIVE PCR

Total RNA was isolated from frozen gastric mucosal biopsy samples using TRIzol separation. cDNA was prepared using the abovementioned method. Real time quantitative RT-PCR has been described previously.^{27–29} All PCR

reactions were performed with a hot start in triplicate in a GeneAmp 5700 Sequence Detection System (Applied Biosystems, Foster City, California, USA). The reaction conditions were two minutes at 50°C, 10 minutes at 95°C, 40 cycles with 15 seconds at 95°C, and a final one minute incubation at 60°C and a final one minute for all templates.

The GeneAmp 5700 system (Applied Biosystems, Foster City, California, USA) detected PCR product accumulation using SYBR Green I assay. SYBR Green I dye was added to bind the double stained DNA formed during the PCR reaction, and binding causes an increase in fluorescence. Based on SYBR Green I assay chemistry, the increase in fluorescence is proportional to the DNA concentration. All PCR reactions were performed in special optical tubes in a 96 well microtitre plate format. Serial dilutions of cDNA standards in triplicate with known amounts of input cDNA and “no template” negative controls were added to the microwells to perform PCR. Intra- and interassay variability were less than 3 %. The transcripts of β-actin were quantified as endogenous RNA control. Then, the target gene amount was divided by the endogenous β-actin amount to obtain a normalised target gene value.

STATISTICAL ANALYSIS

The Student's *t* test was used for statistical analysis. Results were considered significant at *p*<0.05.

Results

CELL GROWTH AND APOPTOSIS

Treatment with *H pylori* markedly inhibited the growth of AGS cells. However, little or no cell death was detected within 24 hours by trypan blue exclusion assay. Extending the incubation time to 72 hours caused half of the *H pylori* treated cells to become detached and resulted in a decrease in the number of living cells. Therefore, AGS cells were treated with *H pylori* for 24 hours in this study.

Spontaneous apoptosis occurred in 2–4% of exponentially growing AGS cells after a 24 hour incubation period, as assessed by subdiploid DNA peak analysis on flow cytometry. Addition of *H pylori* induced a threefold increase in the percentage of cells in the sub-G₁ (apoptotic) population in exponentially dividing cells after 24 hours of incubation (fig 1).

RNA EXPRESSION PATTERN IN *H PYLORI* INFECTED AGS CELLS

The Atlas human cDNA expression array is a positively charged nylon membrane on which DNA fragments representing 588 genes, nine housekeeping genes, and negative control sequences were spotted in duplicate dots. cDNA probes derived from *H pylori* treated AGS cells were hybridised to a membrane. The probes derived from untreated cells were hybridised to another identical membrane. mRNA extracted from AGS cells for cDNA preparation was quantified using a spectrophotometric method (ratio of 260 nm/280 nm). For several genes, changes in expression levels

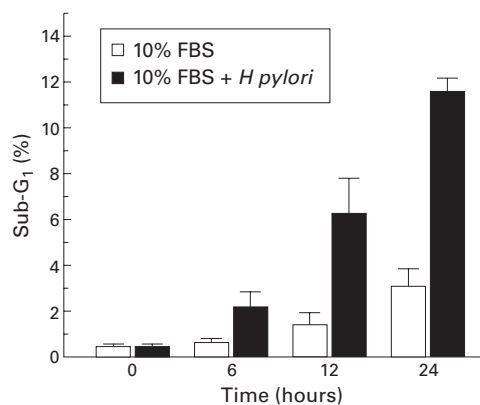


Figure 1 Apoptosis fraction of AGS cells at different times after coculturing with *H pylori*. Synchronised AGS cells were serum deprived for 48 hours (time 0 hours) and fed with fresh medium containing 10% fetal bovine serum (FBS) alone or combined with wild-type *H pylori* at a bacterial concentration of 10^7 cells/ml. The experiments were repeated three times.

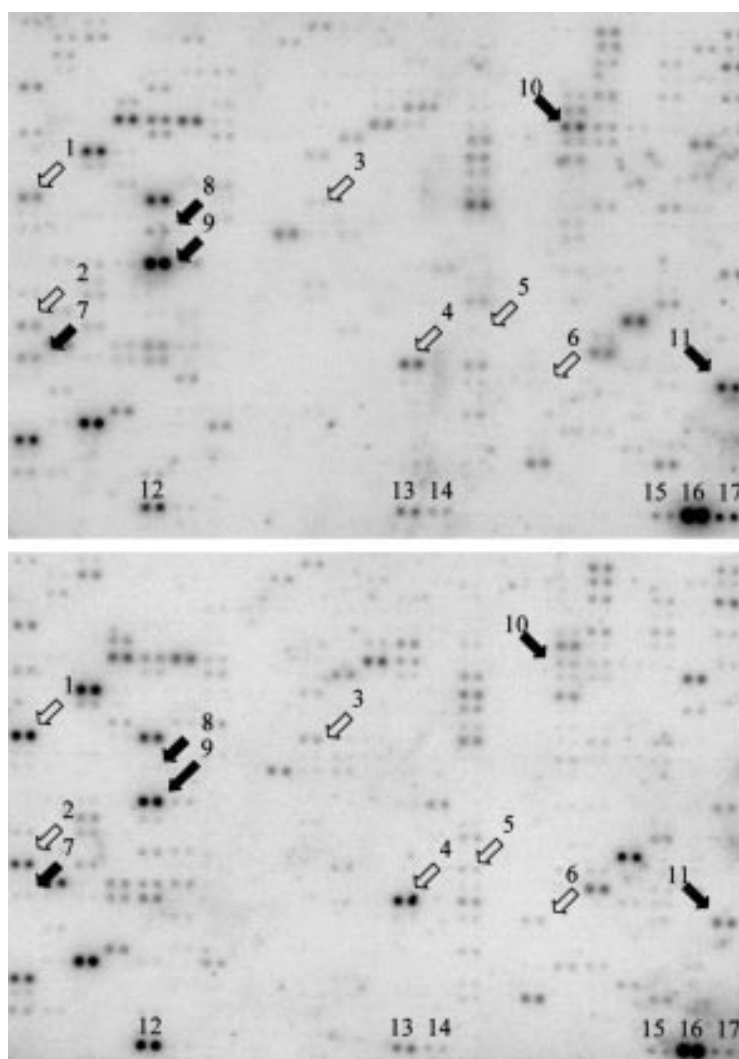


Figure 2 Gene expression pattern after 24 hours of *H pylori* cocultured AGS cells appearing on the Atlas human cDNA expression arrays. Upper and lower arrays were hybridised with the cDNA probes derived from untreated and *H pylori* treated AGS cells, respectively. Some of the differentially expressed genes are marked. Open arrows indicate genes that were overexpressed by *H pylori* treatment; black arrows indicate downregulated genes. 1, *c-jun*; 2, BTEB2; 3, MAPk/ERK kinase; 4, α -catenin; 5, fibronectin receptor α subunit; 6, epidermal growth factor; 7, *Id-2*; 8, *p55CDC*; 9, nuclease sensitive element DNA binding protein; 10, NM23-H2; and 11, death associated protein 3. Internal controls were: 12, ubiquitin; 13, *gapdh*; 14, tubulin; 15, β -actin; 16, 23 kDa highly basic protein; and 17, ribosomal protein S9.

after *H pylori* infection were identified by comparing hybridisation patterns on the two membranes (fig 2). We stripped and rehybridised the cDNA array membranes at least three times and found similar results. Table 1 shows that 21 genes were upregulated and 17 genes were downregulated after AGS cells were cocultured with *H pylori*. Only those genes demonstrating greater than twofold alterations are listed. Nine housekeeping genes were used as internal controls to correct the mRNA abundance. Among these housekeeping genes, *gapdh*, tubulin α , β -actin, gene of 23 kDa highly basic protein, and gene of ribosomal protein S9, which showed similar relative intensities of signals in both hybridised membranes, and the mean of the signals of those control genes were used to normalise the target genes. The signals of other housekeeping genes (*hpert*, phospholipase A2, and MHC) were either not detected or too weak to act as a useful reference. No detectable signal appeared at the sites of M13, λ -DNA, and pUC18 DNA, which served as negative controls for DNA contamination of sample.

NORTHERN BLOT ANALYSIS

To confirm the differential expression of genes identified on the cDNA expression arrays, an independent experiment of *H pylori* treatment was repeated, and total RNAs derived from 24 hour *H pylori* treated and untreated AGS cells were subjected to RT-PCR preparation for six probes (*c-jun*, *CDC25B*, *ERCC3*, *Id-2*, *p55CDC*, and *NM23-H2*). Northern blot analysis revealed that sizes of *c-jun*, *CDC25B*, *ERCC3*, *Id-2*, *p55CDC*, and *NM23-H2* were approximately 4.8, 4.8, 5.6, 5.8, 4.6, and 3.7 kb, respectively. As shown in fig 3, the time course of northern blotting confirmed that candidate gene expression in AGS cells was changed by *H pylori* treatment. In untreated AGS cells, *c-jun* mRNA levels were decreased at 24 hours. However, expression of *c-jun* was maintained for 24 hours in *H pylori* treated AGS cells. *CDC25B* levels in the control AGS cells were decreased at 12 hours and recovered at 24 hours, but in *H pylori* treated AGS cells gene expression was unchanged after 24 hours. *ERCC3* and *P55CDC* expression was significantly decreased at 24 hours by *H pylori* treatment. *Id-2* expression was decreased within 12 hours of *H pylori* treatment and recovered at 24 hours. *NM23-H2* was significantly decreased within 12 hours of *H pylori* treatment and was quantified at 250% of control levels.

QUANTITATIVE RT-PCR ANALYSIS OF CANDIDATE GENES FROM H PYLORI INFECTED AND UNINFECTED GASTRIC MUCOSA

A significant decrease in *ERCC3* mRNA levels was observed in the group of patients with *H pylori* infected gastric mucosal biopsies (mean 4.75 mRNA molecules/ 10^4 β -actin mRNA molecules; range 1.4–8.5) compared with uninfected mucosa (mean 13.65 mRNA molecules/ 10^4 β -actin mRNA molecules; range 8.4–25.9) ($p < 0.001$) (fig 4). A significant decrease in *Id-2* mRNA levels was also observed in *H pylori* infected gastric mucosal

Table 1 Alteration of gene expression in AGS cells after cocultivation with *Helicobacter pylori* for 24 hours

Accession No	Gene or protein	Fold
Overexpressed genes		
J04111	<i>c-jun</i>	5.0
D14520	<i>BTEB 2</i>	3.3
M75914	<i>Interleukin 5 receptor α</i>	3.3
U02368	<i>PAX3/forkhead fusion protein</i>	3.2
M92843	<i>Zinc finger transcription regulator TTP</i>	2.9
X75342	<i>shb src-2 homolog</i>	2.8
L05624	<i>MAPK/ERK kinase 1</i>	2.8
D28118	<i>DB1</i>	2.8
M62831	<i>ETR101</i>	2.7
X16707	<i>fra-1</i>	2.5
M62829	<i>ETR103</i>	2.5
D13866;	<i>α-Catenin</i>	2.5
L32976	<i>MLK-3</i>	2.3
X06374	<i>PDGF-A</i>	2.3
M36429	<i>Transducin β-1 subunit</i>	2.2
U02081	<i>NET1</i>	2.2
X15219	<i>sno oncogene snoN protein ski-related</i>	2.1
L36719	<i>MAP kinase kinase 3</i>	2.0
L36870	<i>MAP kinase kinase 4</i>	2.0
X06256	<i>Fibronectin receptor α subunit</i>	2.0
X04571	<i>Epidermal growth factor</i>	2.0
Downregulated genes		
M97796	<i>Helix-loop-helix protein Id-2</i>	6.3
U05340	<i>p55CDC</i>	5.6
M31899	<i>ERCC3; TFIID 89-kDa subunit</i>	5.6
L16785	<i>NM23-H2</i>	4.9
M64673	<i>Heat shock factor protein 1</i>	4.2
M34960	<i>Transcription factor IID</i>	3.9
L12579	<i>CCAAT displacement protein</i>	3.2
M81934	<i>GDC25B</i>	3.1
D21235	<i>XPAC; HHR23A</i>	3.1
M35410	<i>Insulin-like growth factor binding protein 2</i>	2.9
U18321	<i>Death associated protein 3 (DAP-3)</i>	2.9
X79389	<i>Glutathione S-transferase T1</i>	2.8
U04847	<i>Ini1</i>	2.8
M14200	<i>Diazepam binding inhibitor</i>	2.6
X54079	<i>Heat shock protein HSP27</i>	2.4
L22474	<i>Bax β</i>	2.2
M83234	<i>Nuclease sensitive element DNA binding protein</i>	1.8

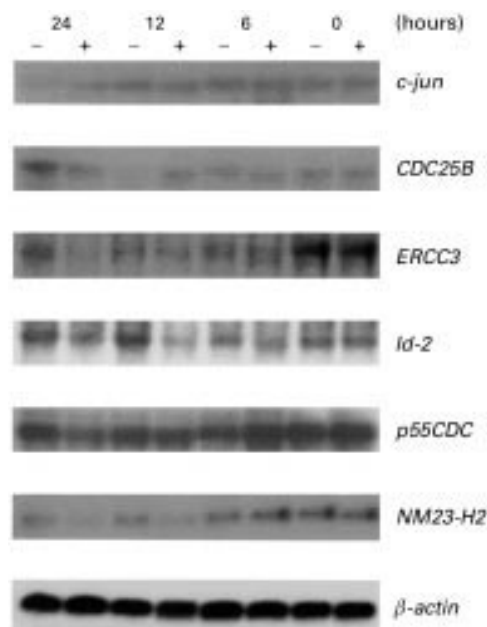


Figure 3 Northern blot demonstrating changes in expression of *ERCC3*, *Id-2*, *NM23-H2*, *c-jun*, and *p55CDC* at specified time intervals after coculturing AGS cells with *H pylori* at 10^7 cells/ml for 24 hours. RNAs were purified at the indicated times, and 10 μ g of total cellular RNA was loaded into each lane. The same blot was hybridised to one probe, retrieved, and rehybridised sequentially with the other probes. Three independent experiments were performed and similar results were obtained.

biopsies (mean 16.1 mRNA molecules/ 10^4 β -actin mRNA molecules; range 10.4–19.3) compared with normal mucosa (mean 23.4 mRNA molecules/ 10^4 β -actin mRNA molecules; range 20.1–35.6) ($p < 0.05$). Expression of *NM23-H2* was significantly decreased in *H pylori* infected gastric mucosal biopsies (mean 17.5 mRNA molecules/ 10^4 β -actin mRNA molecules; range 10.4–19.3) compared with normal mucosa (mean 45.5 mRNA molecules/ 10^4 β -actin mRNA molecules; range 30.1–60.6) ($p < 0.001$). Although in *H pylori* colonised mucosa *CDC25B* and *c-jun* mRNA levels were altered, no significant difference was seen in gene expression between infected and uninfected mucosa (fig 4).

Discussion

Cell lines or primary cultures coincubated with bacterium or treated with bacterial components are frequently used as a model to study the pathogenic mechanisms of *H pylori* related diseases. The cellular responses induced by such treatment include growth inhibition, vacuolation, apoptosis, cytokine secretion, and rearrangement of the cytoskeleton.^{7 30–32} However, it is still difficult to establish a direct link with *H pylori* induced diseases from these effects, and the possibility that other mechanisms may be responsible for the diseases cannot be excluded. Therefore, a comprehensive investigation of the cellular responses to *H pylori* is necessary.

In this study, we used cDNA expression array to monitor expression of hundreds of genes in AGS cells after coincubation with *H pylori* and found that there were changes in expression levels of 38 genes. The majority of the genes were transcription factors or putative transcription factors. Among them *c-jun*, *fra-1*, *PAX3*, *BTEB2*, *TTP*, *DB1*, *ETR101*, and *ETR103* were overexpressed and *Id2*, *TFIID*, *TFIIH-p89*, *NM23-H2*, *Ini1*, *HSF1*, and gene of *CCAAT* displacement protein were down-regulated. Differential expression of transcription factors may be an early response to *H pylori* infection because these factors influence expression of other genes and then change cellular behaviour. Among the transcription factors with elevated expression, C-JUN and Fra-1 are two components of the transcription factor AP-1. AP-1 has a binding site in the promoter region of *IL-8*, and expression of *IL-8* could be partially affected by AP-1.³³ Several lines of evidence have proved that *IL-8* production by cells was stimulated by *H pylori* treatment.^{7–9} Moreover, an increase in AP-1 activity is an early event in cell apoptosis.³⁴ Since prolonging the incubation time of AGS cells with *H pylori* (over 96 hours) causes cell death, AP-1 activity may play a role in *H pylori* induced cell death. Interestingly, some of the *H pylori* induced transcription factors, such as *c-jun*, *TTP*, *ETR101*, and *ETR103*, are also mitogen inducible,^{35–38} suggesting that at least part of the *H pylori* induced response is shared with mitogen mechanisms. In contrast, *TFIID* and *ERCC3*, which are associated with basic transcription, were downregulated by *H pylori*.

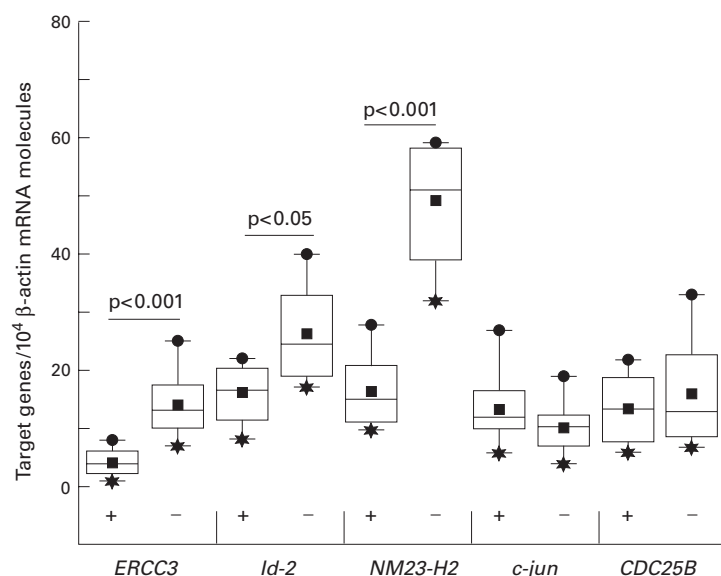


Figure 4 mRNA levels of *ERCC3*, *Id-2*, *NM23-H2*, *c-jun*, and *p55CDC* in human gastric mucosal biopsies. mRNA amounts were quantified using a real time quantitative reverse transcription-polymerase chain reaction technique, as described in patients and methods. For each group data were summarised using the box plot technique. The bottom and top symbols indicated minimum and maximal values, respectively (lower or higher than 10% and 90% percentiles); the filled square in the box indicates the mean; and the bottom of the box, median line, and top of the box indicate 25%, 50%, and 75% percentiles, respectively. + and - indicate *H pylori* infected and uninfected biopsies, respectively.

This phenomenon may be due to the inhibited growth of the cells after *H pylori* treatment.

Some signal transduction pathways can change the activities of transcription factors and their expression. Although the signalling mechanism was not observed directly in the present study, we found clues from the differential expression of the related genes. For example, our results indicated that expression of *MLK-3*, *MAPKK3*, *MAPKK4*, *ERK kinase 1*, and *c-jun* increased after cells were treated with *H pylori*, indicating that the MAP kinase pathway plays a role in *H pylori* induced responses. In contrast, expressions of interleukin 5 receptor (*IL-5R*) and insulin-like growth factor binding protein 2 (*IGFBP2*), which modulate the effects of IL-5 and insulin-like growth factor, respectively, were suppressed by the bacterium. While the physiological inference of *IL-5R* and *IGFBP2* is unclear, cells may change their susceptibility to stimuli by altering the number of factors that are involved in signalling pathways.

Two cell cycle related genes, *CDC25B* and *p55CDC*, were downregulated by *H pylori* infection. *CDC25B*, a tyrosine phosphatase, is required for cell cycle progression to mitosis.³⁹ It has been reported that phorbol 12-myristate 13-acetate suppresses expression of *CDC25B* and causes G2 arrest in endothelial cells.⁴⁰ Our results showed that *H pylori* inhibited cell growth but did not cause cell death during the coinubation period of 24 hours. This growth inhibition may be related to downregulation of *CDC25B*, as evidenced by the fact that coinubation of AGS cells with *H pylori* caused G2 arrest (unpublished observation).

The microtubule association protein *p55CDC* is essential for cell division.⁴¹ Depletion of *p55CDC* by antibody injection causes

arrest or delay at the metaphase and impairs progression of late mitotic events.⁴² Downregulation of *CDC25B* and *p55CDC* suggests that *H pylori* disturbs the cell cycle. Previous studies have confirmed that before apoptosis, UV treated cells suffer cell cycle arrest.⁴³ This raises the question of whether *H pylori* induces apoptosis via a similar mechanism. Downregulated levels of *NM23-H2*, a metastasis suppressor gene, were also observed in *H pylori* treated AGS cells. Increasing evidence suggests that *NM23* genes, initially documented as suppressors of the invasive phenotype in some cancer types, are involved in the control of normal development and differentiation.⁴⁴

H pylori suppressed two stress inducible genes, the heat shock protein 27 and the heat shock factor protein 1, and a detoxification related gene, glutathione S-transferase T1. As expected, *H pylori* can potentially generate reactive oxygen molecules^{16,17} which may cause a stress environment in cells and induce stress genes. However, induction of stress genes was not observed in this study. It may be that oxidative stress was not generated under our cell culturing conditions or that the cellular response to the *H pylori* insult was not mediated by the stress proteins tested.

Using cDNA expression arrays to investigate gene expression, we found that for several genes in AGS cells, expression levels were changed after treatment with *H pylori*. This clearly indicates that multiple genetic changes and gene products are required for the pathogenesis of *H pylori* infection. Using the technique of gene specific quantitative RT-PCR, we demonstrated that the expression patterns of *ERCC3*, *Id-2*, and *NM23-H2* genes analysed in human gastric mucosal biopsies were the same as those using the cDNA expression arrays. Expression of *c-jun* and *p55CDC* in *H pylori* infected and uninfected human gastric mucosa were different but not significant. The possible reason for this is either false positive results from the cDNA expression array assay or scarce amounts of those genes present in the gastric mucosa. We are currently analysing expression patterns of the rest of the target genes that were screened using this technique in *H pylori* colonised human gastric mucosa.

We conclude that differential hybridisation of human cDNA expression arrays can be a useful tool to identify potential genes involved in the pathogenic mechanism of *H pylori*.

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- Solnick JV, Tompkins LS. *Helicobacter pylori* and gastrointestinal disease: pathogenesis and host-parasite interaction. *Infect Agents Dis* 1992;1:284-309.
- Blaser MJ. *Helicobacter pylori*: microbiology of a "slow" bacterial infection. *Trends Microbiol* 1993;1:255-60.
- Hazell SL, Mendz GL. The metabolism and enzymes of *Helicobacter pylori*: function and potential virulence effects. In: Goodwin CS, Worsley BW, ed. *Helicobacter pylori: biology and clinical practice*. Boca Raton: CRC Press, 1993: 115-41.
- Cover TL, Dooley CP, Blaser MJ. Characterization of and human serologic response to proteins in *Helicobacter pylori* broth culture supernatants with vacuolizing cytotoxin activity. *Infect Immun* 1990;58:603-9.

- 5 Piotrowski J. Lipopolysaccharide a virulence factor of *Helicobacter pylori*: effect of antilucer agents. *J Physiol Pharmacol* 1998;**49**:3–24.
- 6 Censini S, Lange C, Xiang Z, et al. Cag, a pathogenicity island of *Helicobacter pylori*, encodes type I-specific and disease-associated virulence factors. *Proc Natl Acad Sci USA* 1996;**93**:14648–53.
- 7 Ogura K, Takahashi M, Maeda S, et al. Interleukin-8 production in primary cultures of human gastric epithelial cells induced by *Helicobacter pylori*. *Dig Dis Sci* 1998;**43**:2738–43.
- 8 Shimada T, Terano A. Chemokine expression in *Helicobacter pylori*-infected gastric mucosa. *J Gastroenterol* 1998;**33**:613–17.
- 9 Gionchetti P, Vaira D, Campieri M, et al. Enhanced mucosal interleukin-6 and -8 in *Helicobacter pylori*-positive dyspeptic patients. *Am J Gastroenterol* 1994;**89**:883–7.
- 10 Lamers CB. Gastric secretory abnormalities in duodenal ulcer: primary or secondary to *Helicobacter pylori* infection? *Scand J Gastroenterol Suppl* 1992;**194**:99–103.
- 11 Valle J, Pikkarainen P, Vuoristo M, et al. *Helicobacter pylori* and duodenal ulcer. A study of duodenal ulcer patients and their first-degree relatives. *Scand J Gastroenterol Suppl* 1991;**186**:45–51.
- 12 Cave TR, Cave DR. *Helicobacter pylori* stimulates pepsin secretion from isolated rabbit gastric glands. *Scand J Gastroenterol Suppl* 1991;**181**:9–14.
- 13 Rudi J, Kuck D, Strand S, et al. Involvement of the CD95 (APO-1/Fas) receptor and ligand system in *Helicobacter pylori*-induced gastric epithelial apoptosis. *J Clin Invest* 1998;**102**:1506–14.
- 14 Jones NL, Shannon PT, Cutz E, et al. Increase in proliferation and apoptosis of gastric epithelial cells early in the natural history of *Helicobacter pylori* infection. *Am J Pathol* 1997;**151**:1695–703.
- 15 Chen G, Sordillo EM, Ramey WG, et al. Apoptosis in gastric epithelial cells is induced by *Helicobacter pylori* and accompanied by increased expression of BAK. *Biochem Biophys Res Commun* 1997;**239**:626–32.
- 16 Pignatelli B, Bancel B, Esteve J, et al. Inducible nitric oxide synthase, anti-oxidant enzymes and *Helicobacter pylori* infection in gastritis and gastric precancerous lesions in humans. *Eur J Cancer Prevent* 1998;**7**:439–47.
- 17 Drake IM, Mapstone NP, Schorah CJ, et al. Reactive oxygen species activity and lipid peroxidation in *Helicobacter pylori* associated gastritis: relation to gastric mucosal ascorbic acid concentrations and effect of *H. pylori* eradication. *Gut* 1998;**42**:768–71.
- 18 Wong L, Lue MY, Chang CA, et al. *Helicobacter pylori* induces gene expression in human gastric cells identified by mRNA differential display. *Biochem Biophys Res Commun* 1996;**228**:484–8.
- 19 DeRisi J, Penland L, Brown PO, et al. Use of a cDNA microarray to analyze gene expression patterns in human cancer. *Nat Genet* 1996;**14**:457–60.
- 20 Martin KJ, Kwan CP, Sager R. In: Ling P, Pardee A, eds. *Methods in molecular biology*. New York: Humana Press, 1997:77–85.
- 21 Shim C, Zhang W, Rhee CH, et al. Profiling of differentially expressed genes in human primary cervical cancer by complementary DNA expression array. *Clin Cancer Res* 1998;**4**:3045–50.
- 22 Sehgal A, Boynton AL, Young RF, et al. Application of the differential hybridization of Adas human expression arrays technique in the identification of differentially expressed genes in human glioblastoma multiforme tumor tissue. *J Surg Oncol* 1998;**67**:234–41.
- 23 Bubendorf L, Kononen J, Koivisto P, et al. Survey of gene amplifications during prostate cancer progression by high-throughout fluorescence in situ hybridization on tissue microarrays. *Cancer Res* 1999;**59**:803–6.
- 24 Khan J, Simon R, Bittner M, et al. Gene expression profiling of alveolar rhabdomyosarcoma with cDNA microarrays. *Cancer Res* 1998;**58**:5009–13.
- 25 Hilsenbeck SG, Friedrichs WE, Schiff R, et al. Statistical analysis of array expression data as applied to the problem of tamoxifen resistance. *J Natl Cancer Inst* 1999;**91**:453–9.
- 26 Debouck C, Goodfellow PN. DNA microarrays in drug discovery and development. *Nat Genet* 1999;**21**:48–50.
- 27 Bieche I, Laurendeau I, Tozlu S, et al. Quantitation of MYC gene expression in sporadic breast tumors with a real-time reverse transcription-PCR assay. *Cancer Res* 1999;**59**:2757–65.
- 28 Gibson UEM, Heid CA, Williams PM. A novel method for real time quantitative RT-PCR. *Genome Methods* 1996;**6**:995–1001.
- 29 Kruse N, Pette M, Toyka K, et al. Quantification of cytokine mRNA expression by RT-PCR in samples of previously frozen blood. *J Immunol Methods* 1997;**210**:195–203.
- 30 Wagner S, Beil W, Westermann J, et al. Regulation of gastric epithelial cell growth by *Helicobacter pylori*: offence for a major role of apoptosis. *Gastroenterology* 1997;**113**:1836–47.
- 31 Cover TL, Halter SA, Blaser MJ. Characterization of HeLa cell vacuoles induced by *Helicobacter pylori* broth culture supernatant. *Hum Pathol* 1992;**23**:1004–10.
- 32 Segal ED, Falkow S, Tompkins LS. *Helicobacter pylori* attachment to gastric cells induces cytoskeletal rearrangements and tyrosine phosphorylation of host cell proteins. *Proc Natl Acad Sci USA* 1996;**93**:1259–64.
- 33 Aihara M, Tsuchimoto D, Takizawa H, et al. Mechanisms involved in *Helicobacter pylori*-induced interleukin-8 production by a gastric cancer cell line, MKN45. *Infect Immun* 1997;**65**:3218–24.
- 34 Jehn BM, Osborne BA. Gene regulation associated with apoptosis. *Crit Rev Eukaryotic Gene Expr* 1997;**7**:179–93.
- 35 Chiariello M, Marinissen MJ, Gutkind JS. Multiple mitogen-activated protein kinase signaling pathways connect the c-myc oncoprotein to the c-jun promoter and to cellular transformation. *Mol Cell Biol* 2000;**20**:1747–58.
- 36 Taylor GA, Thompson MJ, Lai WS, et al. Phosphorylation of tristetraprolin, a potential zinc finger transcription factor, by mitogen stimulation in intact cells and by mitogen-activated protein kinase *in vitro*. *J Biol Chem* 1995;**270**:13341–7.
- 37 Shimizu N, Ohta M, Fujiwara C, et al. Expression of a novel immediate early gene during 12-O-tetradecanoylphorbol-13-acetate-induced macrophagic differentiation of HL-60 cells. *J Biol Chem* 1991;**266**:12157–61.
- 38 Shimizu N, Ohta M, Fujiwara C, et al. A gene coding for a zinc finger protein is induced during 12-O-tetradecanoylphorbol-13-acetate-stimulated HL-60 cell differentiation. *J Biochem* 1992;**111**:272–7.
- 39 Lammer C, Wager S, Saffrich R, et al. The cdc25B phosphatase is essential for the G2/M phase transition in human cells. *J Cell Sci* 1998;**111**:2445–53.
- 40 Kosaka C, Sasaguri T, Ishida A, et al. Cell cycle arrest in the G2 phase induced by phorbol ester and diacylglycerol in vascular endothelial cells. *Am J Physiol* 1996;**270**:C170–8.
- 41 Weinstein J, Jacobsen FW, Hsu-Chen J, et al. A novel mammalian protein, p55CDC, present in dividing cells is associated with protein kinase activity and has homology to the Saccharomyces cerevisiae cell division cycle proteins Cdc20 and Cdc4. *Mol Cell Biol* 1994;**14**:3350–63.
- 42 Kallio M, Weinstein J, Daum JR, et al. Mammalian p55CDC mediates association of the spindle checkpoint protein Mad2 with the cyclosome/anaphase-promoting complex, and is involved in regulating anaphase onset and late mitotic events. *J Cell Biol* 1998;**141**:1393–406.
- 43 Orren DK, Petersen LN, Bohr VA. Persistent DNA damage inhibits S-phase and G2 progression, and results in apoptosis. *Mol Biol Cell* 1997;**8**:1129–42.
- 44 Lombardi D, Lacombe ML, Paggi MG. NM23: unraveling its biological function in cell differentiation. *J Cell Physiol* 2000;**182**:144–9.