Modern Pathology (2006) 19, 49–58
© 2006 USCAP, Inc All rights reserved 0893-3952/06 $30.00
www.modernpathology.org

Altered expression of Skp2, c-Myc and p27 proteins but not mRNA after *H. pylori* eradication in chronic gastritis

Sung S Kim[1,3], Patricia Meitner[2], Tamako A Konkin[2], Young S Cho[3], Murray B Resnick[2] and Steven F Moss[1]

[1]*Department of Medicine, Rhode Island Hospital/Brown University, Providence, RI, USA;* [2]*Department of Pathology, Rhode Island Hospital/Brown University, Providence, RI, USA and* [3]*Department of Internal Medicine, The Catholic University of Korea, Seoul, Korea*

Helicobacter pylori **infection is associated with increased gastric epithelial cell turnover and non-cardia gastric cancer. Cell cycle progression is dependent on the proteasomal degradation of p27, a cyclin-dependent kinase inhibitor and gastric tumor suppressor, following ubiquitination mediated by Skp2. c-Myc is a transcriptional repressor of p27 and also a target of Skp2.** ***In vitro*****,** ***H. pylori*** **decreases p27 protein post-translationally. We aimed to determine how p27 is regulated by** ***H. pylori in vivo*****. The effect of eradicating** ***H. pylori*** **on gastric epithelial p27, Skp2, and c-Myc proteins and mRNA was investigated in 22 patients with chronic gastritis, by immunohistochemistry and laser capture microdissection. The percentage of gastric antral epithelial cells expressing p27 protein was significantly higher after eradication of** ***H. pylori*** **(mean$\pm$s.e.m. 37$\pm$2.4% pre-eradication** ***vs*** **55$\pm$2.8% post-eradication;** ***P*****$<$0.001), while Skp2 and c-Myc protein-expressing cells were lower (Skp2: 35$\pm$3.8** ***vs*** **23$\pm$2.6%,** ***P*****$=$0.009; c-Myc: 47$\pm$3.6** ***vs*** **30$\pm$3.8%,** ***P*****$<$0.001). mRNA expressions of p27, Skp2, and c-Myc (normalized for 18SrRNA) were not changed by** ***H. pylori*** **eradication.** ***H. pylori*** **increases c-Myc and decreases gastric epithelial p27 protein expression in association with increased expression of Skp2, the regulator of p27's ubiquitin ligase complex.** ***H. pylori*** **may influence cell cycle progression and carcinogenesis through post-translational effects on specific gene expression.**
Modern Pathology (2006) **19,** 49–58. doi:10.1038/modpathol.3800476; published online 5 August 2005

Keywords: gastric carcinogenesis; cell cycle; *Helicobacter pylori*; protein regulation; cyclin-dependent kinase inhibitor

Epidemiological, clinico-pathological, and animal studies performed over the last 20 years have provided increasing evidence for the importance of prior infection by the gastric bacterium *Helicobacter pylori* in the genesis of gastric cancer.[1,2] It has been estimated that 59% of gastric cancers worldwide are directly attributable to *H. pylori*.[3] Both host-related genetic susceptibility and bacterial virulence factors contribute to the tendency of *H. pylori* to induce cancer in only a minority of all individuals at risk.[4,5] For the most common histological subtype of gastric cancer (the 'intestinal' subtype[6]), a pre-neoplastic sequence manifests from chronic superficial gastritis through atrophic gastritis, intestinal metaplasia, and dysplasia to cancer, as defined originally by Correa *et al.*[7] Progression through these histological cancer precursors is accompanied by the accumulation of mutations, including that of p53, typical of a multi-step process of carcinogenesis.[8] The 'diffuse' subtype of gastric cancer[6] may also be preceded by years of chronic gastritis,[8] but typically without progression through recognizable atrophic change or metaplasia. Reduction or loss of expression of E-cadherin and catenins by both mutation and CpG hypermethylation are frequently noted molecular changes in diffuse gastric carcinogenesis.[8] Although the precise molecular and cellular events responsible for the promotion of gastric cancer by *H. pylori* remain poorly defined, the ability of *H. pylori* to increase gastric epithelial cell turnover[9] is a potentially important step, based on the widespread association of neoplastic transformation with hyperproliferative states.[10]

$p27^{kip1}$ is a cyclin-dependent kinase inhibitor that binds to cyclinE/cdk2, thereby blocking the G1/S

Correspondence: Dr SF Moss, MD, Division of Gastroenterology, Department of Medicine, Rhode Island Hospital, 593 Eddy St, APC 414, Providence, RI 02903, USA.
E-mail: Steven_Moss@Brown.edu
Received 15 March 2005; revised and accepted 1 July 2005; published online 5 August 2005

transition necessary for cell cycle progression.[11] The level of p27 is upregulated under stress conditions, leading to cell cycle inhibition and apoptosis.[11] Additional roles for p27 have also been proposed recently, including tumor suppression[12] and the regulation of cell migration[13] and mitosis.[14] p27 protein levels are mainly regulated through degradation by ubiquitin-dependent proteolysis.[15] Protein ubiquitination requires a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2), and a ubiquitin ligase, or E3.[16] The E3 ligase activity for p27 is provided by the SCF complex, composed of Skp1, cullin-1, rbx-1, and the p27-specific F-box protein Skp2.[16] Skp2 specifically interacts with the extreme COOH-terminus of p27 only when Thr-187 is phosphorylated by cyclin E/CDK2.[17,18] This association of Skp2 with p27 results in the recruitment of p27 to the SCF core complex, thereby promoting its ubiquitination and degradation.[19] Mice lacking Skp2 accumulate p27, suggesting a pivotal role for Skp2 in the degradation of p27 *in vivo*.[20] However, other reports suggest the existence of an Skp2-independent mechanism of p27 degradation.[21]

Low p27 expression has been reported in many cancers, including those of the colon, breast, prostate, lung, and brain, as well as in gastric carcinoma of both intestinal and diffuse subtypes.[11,22–24] Decreased p27 in cancer is associated with a poorer prognosis.[23,25] p27 protein is also decreased in *H. pylori* infection, both in areas of gastritis and in intestinal metaplasia.[26,27] *In vitro*, the addition of *H. pylori* to cultured gastric epithelial cells increases proteasome-dependent p27 protein degradation without altering p27 gene transcription.[28] However, it is unknown whether similar mechanisms occur *in vivo* or whether decreased p27 is restored to normal following *H. pylori* eradication in chronic gastritis.

Skp2 is upregulated and is also related to a poor prognosis in gastric carcinoma,[29] but it remains uncertain whether increased Skp2 mediates accelerated degradation of p27 protein or whether Skp2 influences p27 through other mechanisms. Skp2 regulates the expression of many intracellular proteins by ubiquitination, including p27, c-Myc, $p57^{kip2}$, Smad4, and E2F-1, thus leading to their increased proteasomal degradation.[20,30–34] The effect of *H. pylori* on Skp2 has not been previously evaluated.

The c-Myc oncoprotein is a basic helix–loop–helix zipper transcriptional factor that regulates several genes intimately involved in cell proliferation, differentiation, apoptosis, and oncogenesis.[35] c-Myc decreases not only transcription but also the nuclear transport of p27 from the cytoplasm, thus regulating the G1/S transition for cell cycle progression through p27.[36,37] The SCF component Skp2 accelerates c-Myc ubiquitination and destruction, as well as stimulates c-Myc transcription in cells progressing to S phase.[30,31] Thus, several mechanisms may regulate p27 expression through c-Myc and Skp2 in the context of *H. pylori* infection, and the ability of Skp2 to both activate c-Myc and destroy p27 may be relevant to the development of *H. pylori*-associated gastric cancer. Thus far, no evidence exists that the p27–Skp2–c-Myc axis is related on a molecular basis or epidemiologically to the established p53 and E-cadherin pathways of gastric carcinogenesis.[8]

Owing to the importance of translating these observations regarding p27, Skp2, and c-Myc derived from *in vitro* cellular models to understand the mechanisms of *H. pylori*-associated gastric carcinogenesis, we investigated whether *in vivo* low p27 levels are reversible after the eradication of *H. pylori*, whether p27 protein in *H. pylori* infection is related to low p27 mRNA expression, and whether low p27 protein expression in *H. pylori* infection is associated with altered expression of Skp2 and c-Myc.

Materials and methods

Patients and Tissue Collection

A total of 22 patients with chronic gastritis who had successfully been treated for *H. pylori* in Uijongbu St. Mary's Hospital, Korea, were included in this study. Their mean age was 53 years (range 33–71); 12 were male. Two endoscopic antral biopsies were taken before and 2 months after eradication of *H. pylori*. Tissues were fixed in neutral-buffered formalin and embedded in paraffin blocks for RNA extraction and immunohistochemical staining. *H. pylori* infection was diagnosed in biopsies by a rapid urease test, H&E staining, and immunohistochemistry. *H. pylori* was eradicated using a proton pump inhibitor (omeprazole, lansoprazole, or rabeprazole) with amoxicillin and clarithromycin for 7–14 days.[38] Eradication was confirmed by a negative urea breath test, and by the assessment of visible *H. pylori* bacteria on H&E staining and immunohistochemistry.[38]

Laser Capture Microdissection (LCM) and RNA Extraction

Formalin-fixed, paraffin-embedded tissues were sectioned at 8 μm thickness for RNA extraction. The sections were deparaffinized with xylene, and rehydrated with diluted ethanols in RNase-free water. Slides were then stained with HistoGene staining solution (Arcturus, Mountain View, CA, USA), dehydrated for 30 s in 75, 95%, and for 60 s in anhydrous 100% ethanol serially, and placed in two changes of xylene (5 min each). After 5 min drying at room temperature, 5–8 thousand epithelial cells were microdissected using the AutoPix LCM instrument (Arcturus) and harvested onto a capsure Macro

LCM cap (Arcturus). Glands exhibiting intestinal metaplasia were specifically excluded.

RNA was then extracted from the harvested cells using the Optimum FFPE RNA Isolation kit (Ambion, Austin, TX, USA) as recommended in the manufacturer's instructions with modifications. Briefly, cells were digested with 110 μl (1.81 μg/μl) proteinase K at 42°C for 2 h. In all, 100 μl of RNA extraction buffer was then added, and 105 μl of 100% ethanol was admixed with vortexing. This mixture was transferred to the Filter Cartridge Assembly, and centrifuged at 13 200 rpm for 30 s. The cartridge was washed serially with Washing Solutions 1 and 2/3, and 14 μl of RNA was eluted at 75°C. In all, 1 μl of RNA was used to check the concentration using the BioPhotometer (Eppendorf, Germany), and the remaining RNA was stored at −80°C.

mRNA Quantification Using Real-Time Reverse Transcription Polymerase Chain Reaction (PCR)

Total RNA extracted from microdissected cells was treated with RNase-free DNase I (Roche Diagnostics Corporation, Indianapolis, IN, USA) to remove contaminating genomic DNA. Reverse transcription of RNA was performed in a final volume of 20 μl containing 4 μl of 5 × reverse transcription buffer (250 mM Tris-HCl, 40 mM $MgCl_2$, 150 mM KCl, 5 mM dithiothreitol, pH 8.5), 2.5 mM each deoxynucleotide triphosphate, 40 U of RNase inhibitor, 3.2 μg random hexamers, 20 U of avian myeloblastosis virus reverse transcriptase (all from Roche), and 250 ng/10 μl of total RNA. Samples were incubated at 25°C for 10 min and 42°C for 1 h, the reverse transcriptase was then inactivated by heating at 99°C for 5 min, and the samples were kept on ice until use.

Real-time quantitative PCR was performed using the iCycler iQ Multi-Color Real Time PCR Detection System (Bio-Rad, Hercules, CA, USA) in a 25 μl reaction mix containing 5 μl of diluted reverse transcribed samples (5.0 ng of equivalent total RNA), 12.5 μl of QuantiTec™ SYBR Green PCR Master Mix (Qiagen, Valencia, CA, USA), and serially diluted plasmid constructs of pcDNA3-*p27*,[39] pcDNA3-*skp2* (provided by Michele Pagano, New York University, NY, USA), pLXSH-*c-myc*,[40] or pcDNA2.1-*18SrRNA*[41] in the standard curve. cDNA reverse transcribed from RNA from the AGS gastric carcinoma cell line was used to construct the standard curve for *interleukin-8* (*IL-8*). The sequence and specific volume of each primers used are listed in Table 1. Thermocycling was carried out for 45 cycles, with denaturation at 95°C for 15 s, annealing for 30 s at the temperature listed in Table 1, and extension at 72°C for 30 s. Standard serial 10 × dilutions were prepared from the plasmid constructs or the AGS cell line for IL-8 to relate the threshold cycle to the log input amount of template. The relative amounts of gene transcripts for *p27*, *skp2*, *c-myc*, and *IL-8* were determined using the relative standard curve method, and were normalized for *18SrRNA*.[41]

Immunohistochemistry for p27, Skp2, c-Myc, and *H. pylori* and Evaluation of Inflammation

Formalin-fixed, paraffin-embedded antral biopsy specimens were sectioned at 5 μm thickness, and mounted on Fisherbrand Superfrost plus slides (Fisher Scientific, Pittsburgh, PA, USA). After antigen retrieval (for c-Myc in 1 mM EDTA (pH 8.0) and for Skp-2 in 10 mM citrate buffer (pH 6.0)), sections were incubated overnight at 4°C with monoclonal mouse antibodies to c-Myc (1:100 dilution, Abcam Inc., Cambridge, MA, USA), Skp-2 (1:30 dilution, Zymed, San Francisco, CA, USA), or $p27^{kip1}$ (1:100 dilution, Zymed) or rabbit polyclonal anti-*H. pylori* antibody (1:500 dilution, DakoCytomation, Carpinteria, CA, USA). Immunoreactivity was detected using the DAB Map Kit (PIERCE, Woburn, MA, USA), based on the avidin–biotin complex immunoperoxidase technique.

Positively stained cells were evaluated using image analysis (Image-Pro Plus, version 4.5.1, Media Cybernetics, Silver Spring, MD, USA) to reduce observer variation. After thresholding, positively

Table 1 PCR primers used in this study

Gene	*Primer sequences*	*Annealing temp (°C)*	*Product size (bp)*
p27	F: 5′-AGGACACGCATTTGGTGGA-3′ R: 5′-TAGAAGAATCGTCGGTTGCAGGT-3′	61	108
skp2	F: 5′-GAAACGGCTGAAGAGCAAAG-3′ R: 5′-GAAGGGAGTCCCATGAAACA-3′	60	101
c-myc	F: 5′-TCAAGAGGCGAACACACAAC-3′ R: 5′-GGCCTTTTCATTGTTTTCCA-3′	60	110
18SrRNA	F: 5′-GGACACGGACAGGATTGACA-3′ R: 5′-ACCCACGGAATCGAGAAAGA-3′	60	50
IL-8	F: 5′-GCCAACACAGAAATTATTGTAAAGCTT-3′ R: 5′-AATTCTCAGCCCTCTTCAAAAACTT-3′	54	112

F: forward primer, R: reverse primer.

stained nuclei were counted in a total of 200 epithelial cells in each compartment of the gastric gland (surface, neck, and basal portion), and expressed as a percentage for each compartment and for the entire gland. Acute and chronic inflammation and the *H. pylori* colonization density of chronic gastritis were scored by Resnick using the updated Sydney system.[42]

Statistics

Differences in mRNA and/or protein expression of p27, Skp2, c-Myc, and IL-8 before and after *H. pylori* eradication were compared by Wilcoxon's signed rank test. Correlations between expression of these genes and gastric inflammation in the pre-eradication values were analyzed using Spearman's correlation coefficient. A *P*-value less than 0.05 was regarded as statistically significant.

Results

Histology

In all, 19 of the 22 patients had atrophic gastritis (11 moderate, eight mild) with intestinal metaplasia in 12 cases. Only three patients had neither atrophy nor intestinal metaplasia. After *H. pylori* eradication, scores of chronic inflammation were significantly lower and scores of acute inflammation and *H. pylori* colonization were zero in all cases (Table 2).

Immunohistochemistry of p27, Skp2, and c-Myc

p27, Skp2, and c-Myc immunohistochemistry resulted in dark brown nuclear staining patterns in the epithelial and interstitial inflammatory cells of the gastric antral mucosa. Expression of p27 protein in gastric epithelial cells was higher after the eradication of *H. pylori* (Figure 1a and b). In contrast, the percentages of Skp2 (Figure 1c and d) and c-Myc (Figure 1e and f) positive epithelial cell nuclei were significantly lower after *H. pylori* eradication. Summarizing the data for the entire group of 22 patients, the percentage of gastric antral epithelial cells expressing p27 protein was significantly higher after the eradication of *H. pylori* (mean $\pm$ s.e.m. $37 \pm 2.4\%$ pre-eradication *vs* $55 \pm 2.8\%$ post-eradication; $P < 0.001$) (Figure 2a). In contrast, Skp2 protein expression in these same cells was lower after *H. pylori* eradication (35 ± 3.8 *vs* $23 \pm 2.6\%$, $P = 0.009$) (Figure 2b), and so was the expression of c-Myc (47 ± 3.6 *vs* $30 \pm 3.8\%$, $P < 0.001$) (Figure 2c). No differences were observed in expression of p27, Skp2, or c-Myc either before or after the eradication of *H. pylori* between cases with atrophic gastritis and the three cases with non-atrophic gastritis. The changes in p27 and c-Myc protein following *H. pylori* eradication were most evident in epithelial cells of the glandular surface (for p27: $44 \pm 4.1\%$ pre-eradication *vs* $67 \pm 4.1\%$ post-eradication; $P < 0.001$, for c-Myc: 44 ± 4.9 *vs* $26 \pm 4.9\%$; $P = 0.02$, respectively) and in the neck (p27: 27 ± 3.0 *vs* $43 \pm 3.0\%$; $P = 0.002$, c-Myc: 56 ± 4.5 *vs* $32 \pm 4.1\%$; $P < 0.001$, respectively). Skp2 was significantly lower only in the neck of the gastric glands ($36 \pm 4.3\%$ pre-eradication *vs* $31 \pm 2.1\%$ post-eradication; $P = 0.03$).

Table 2 Scores of inflammation and *H. pylori* colonization in gastric mucosa (mean $\pm$ s.e.m.)

	Before eradication	*After eradication*	P
Acute inflammation	1.5 ± 0.11	0	0.000
Chronic inflammation	1.7 ± 0.13	1.1 ± 0.11	0.005
H. pylori density	2.2 ± 0.15	0	0.000

Scores were determined using the updated Sydney system of grading gastritis.[42] Differences between values before and after eradication were compared by Wilcoxon's signed rank test.

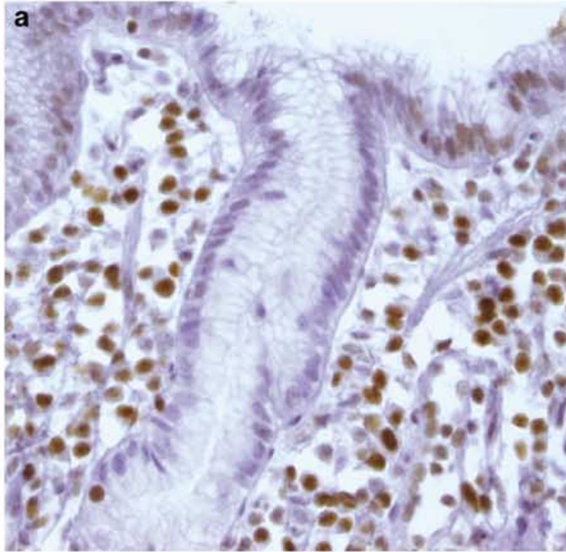
LCM and mRNA Quantification

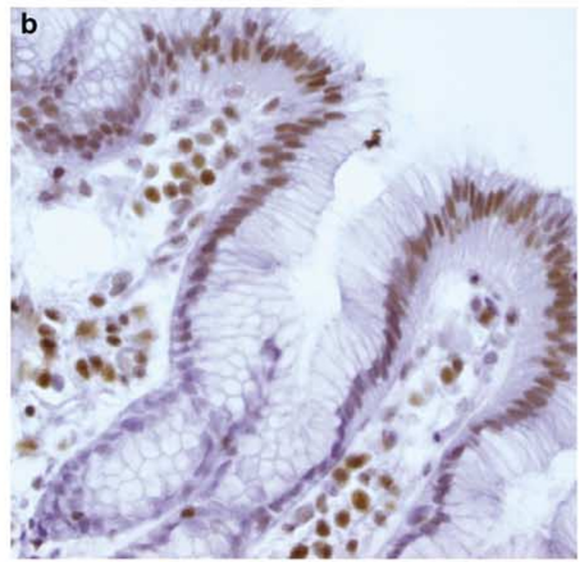
LCM of epithelial cells was successful from a single 8-μm section in all specimens, and yielded on average 441 ng RNA from 5000–8000 epithelial cells (Figure 3). IL-8 mRNA was significantly lower after eradication of *H. pylori* (the mean $\pm$ s.e.m. *IL-8* mRNA/18SrRNA ratio was 111 ± 25.1 before *vs* 16 ± 5.3 after eradication, $P < 0.001$) (Figure 4); this fall in IL-8 is consistent with previous reports.[43,44] The *IL-8* mRNA pre-eradication value correlated significantly with histological scores of acute inflammation ($R = 0.55$, $P = 0.004$). In contrast, mRNA levels of *p27*, *skp2*, and *c-myc* were not altered significantly by *H. pylori* eradication. The mRNA expression of *p27*, *skp2*, and *c-myc* (normalized by 18SrRNA) was not significantly different between before and after eradication of *H. pylori* (*p27* mRNA 19 ± 2.6 *vs* 23 ± 5.1, $P = 0.26$, *skp2* mRNA 33 ± 6.2 *vs* 27 ± 5.1, $P = 0.34$, and *c-myc* mRNA 44 ± 6.2 *vs* 49 ± 14.1, $P = 0.72$, respectively) (Figure 5). There was no correlation between mRNA expression and protein expression in *Helicobacter*-associated gastritis for each gene evaluated, nor was there a difference between cases with atrophic gastritis and cases without atrophy.

Figure 1 Immunohistochemistry for p27, Skp2, and c-Myc. p27, Skp2, and c-Myc proteins were detected in the nuclei of epithelial and interstitial inflammatory cells of the gastric mucosa. Expression of p27 protein was higher in gastric antral epithelial cells after the eradication of *H. pylori*. In contrast, the expression of both Skp2 and c-Myc proteins was lower after the eradication of *H. pylori*. Original magnification: $\times 400$.

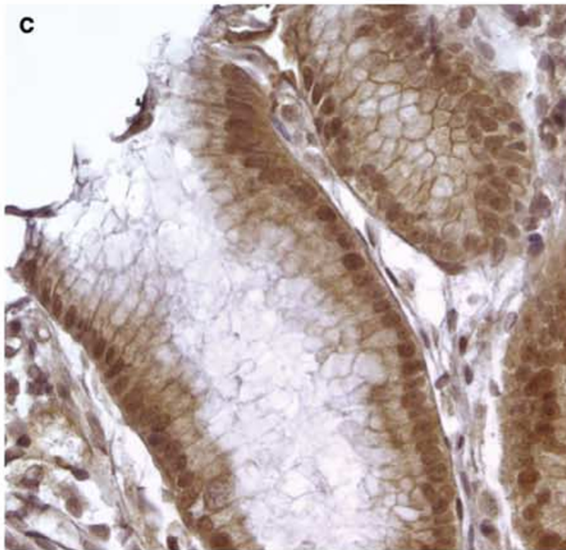
H. pylori	+	-

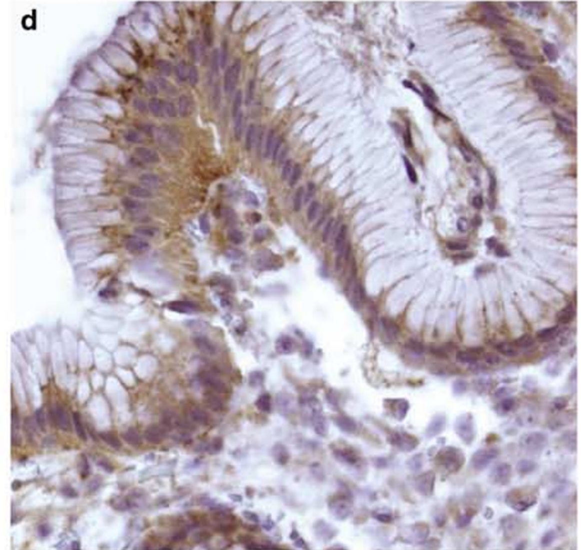
p27



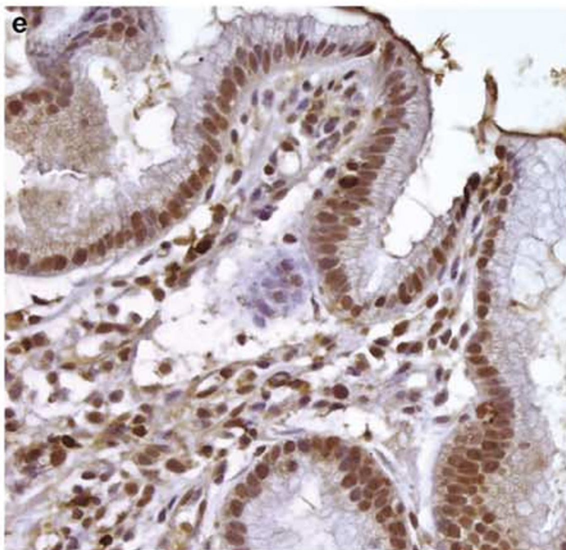


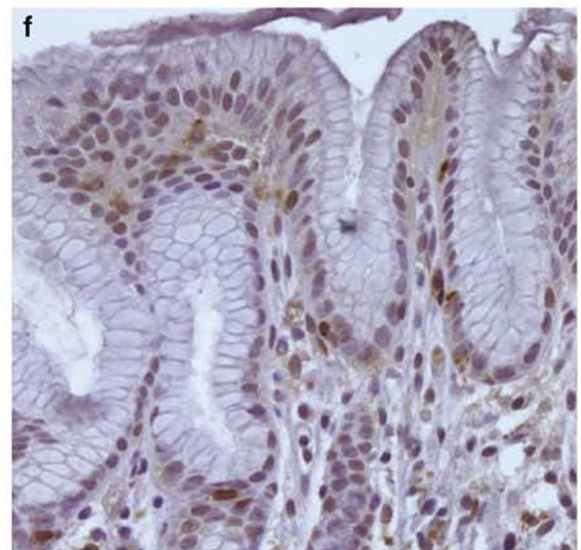
Skp2





c-Myc





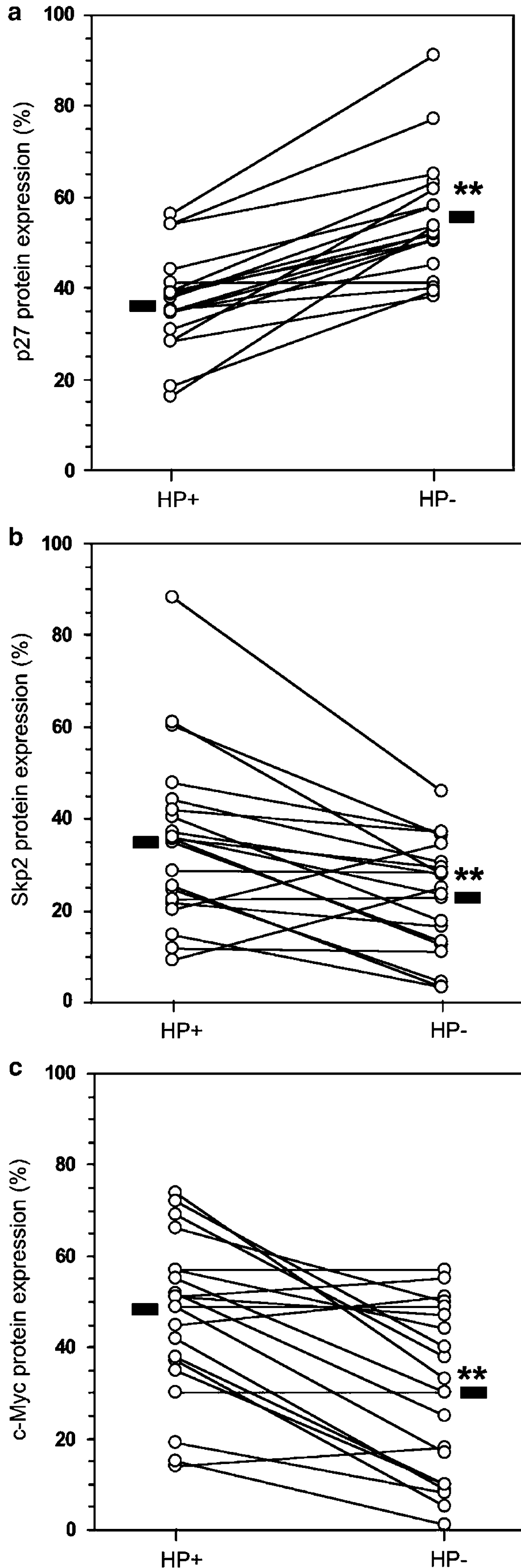


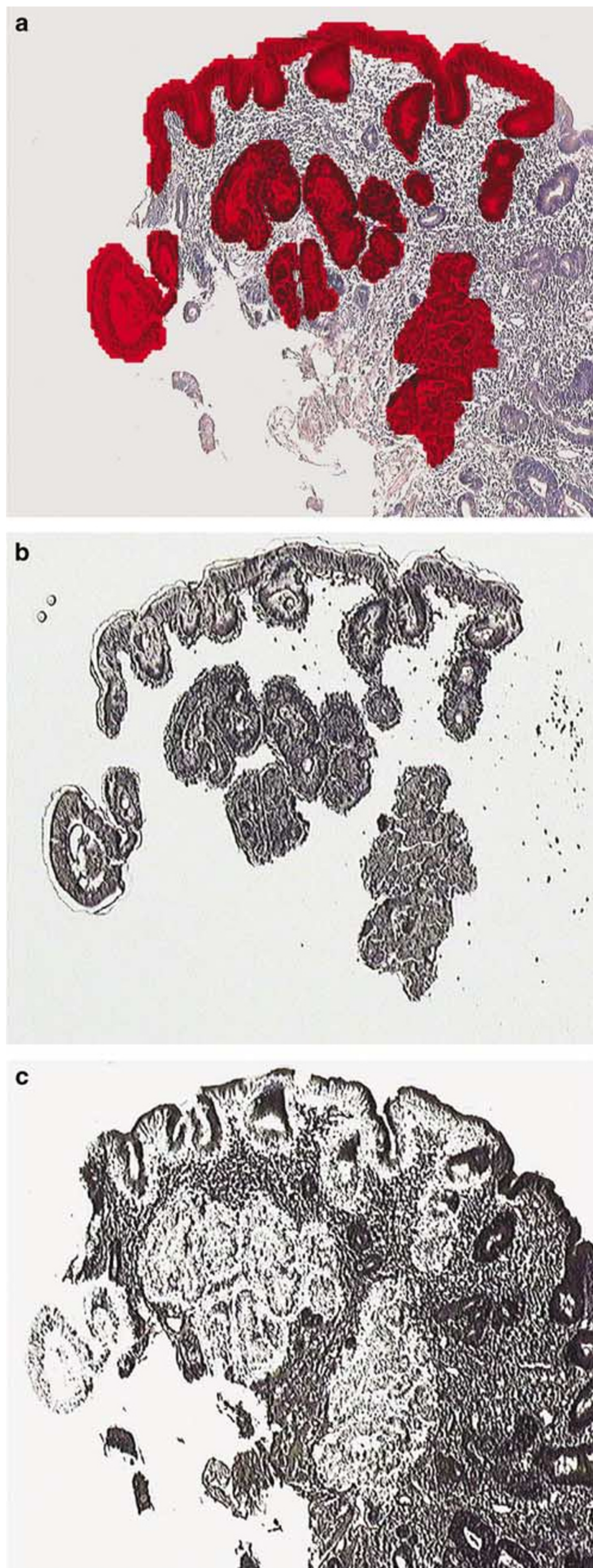
Figure 2 Changes in expression of p27, Skp2, and c-Myc proteins in gastric antral epithelial cells of 22 patients following the eradication of *H. pylori*. The percentage of epithelial cells expressing p27 protein was significantly higher after eradication of *H. pylori* (**a**). Gastric epithelial cells expressing Skp2 (**b**) or c-Myc (**c**) were significantly lower after *H. pylori* eradication. The percentage of epithelial cells expressing each specific protein was determined using image analysis software on representative areas of well-oriented immunostained sections. The thick black bars indicate the mean values, $^{**}P<0.05$.

Discussion

The main findings of our study are that the decrease in epithelial expression of p27 associated with *H. pylori* is reversible following *H. pylori* eradication, and that this decrease in p27 associated with chronic *H. pylori*-associated gastritis is mainly at the level of p27 protein, without significant change in p27 mRNA. These findings are consistent with the major effect of *H. pylori* on p27 observed *in vitro*, namely that it decreases p27 expression through increasing p27 protein degradation.[28] In co-culture, *H. pylori* alone produces this effect, suggesting that the decrease observed *in vivo* is due to the bacterium itself rather than the associated inflammatory response present in the gastric mucosa. However, the effect of *H. pylori* on p27 expression may be modulated by inflammatory cells and cytokines present in the gastric mucosa colonized by *H. pylori*. Although p27 was reported to be increased in the proliferative zone of gastric glands,[45] we found that this region expressed very little p27 compared to both the epithelial surface and the glandular base.

The expression of Skp2, the ubiquitin ligase responsible for p27 degradation during cell cycle progression, is increased in many cancers, including gastric cancer.[29] We also found a reciprocal relationship between low p27 and elevated Skp2 expression in our study. While p27 increased following the eradication of *H. pylori*, Skp2 decreased. As with p27, the change in expression level was confined to Skp2 protein and not mRNA, which again favors a predominantly post-translational level of regulation for Skp2, consistent with prior *in vitro* studies.[46–48] Whether the inverse relationship between Skp2 and p27 is a causal one remains to be determined, since the reduction of p27 protein by *H. pylori in vitro* was not associated with increased Skp2.[28] However, in co-culture *H. pylori* inhibits cell cycle progression,[49] whereas *in vivo H. pylori*-associated gastritis is a hyperproliferative condition;[9] thus, increased Skp2 in *H. pylori*-infected patients may just reflect increased cell proliferation.

H. pylori infection was associated with higher expression of both Skp2 and c-Myc. c-Myc has previously been reported to be increased in *H. pylori*-associated gastritis, is associated with increased cell proliferation, and, as in our current study, was lower after the eradication of *H. pylori*.[50,51] Increased c-Myc may be responsible for



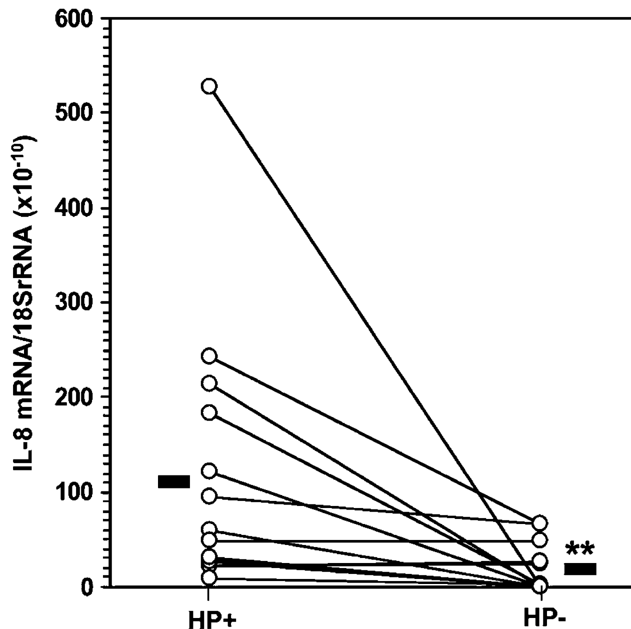
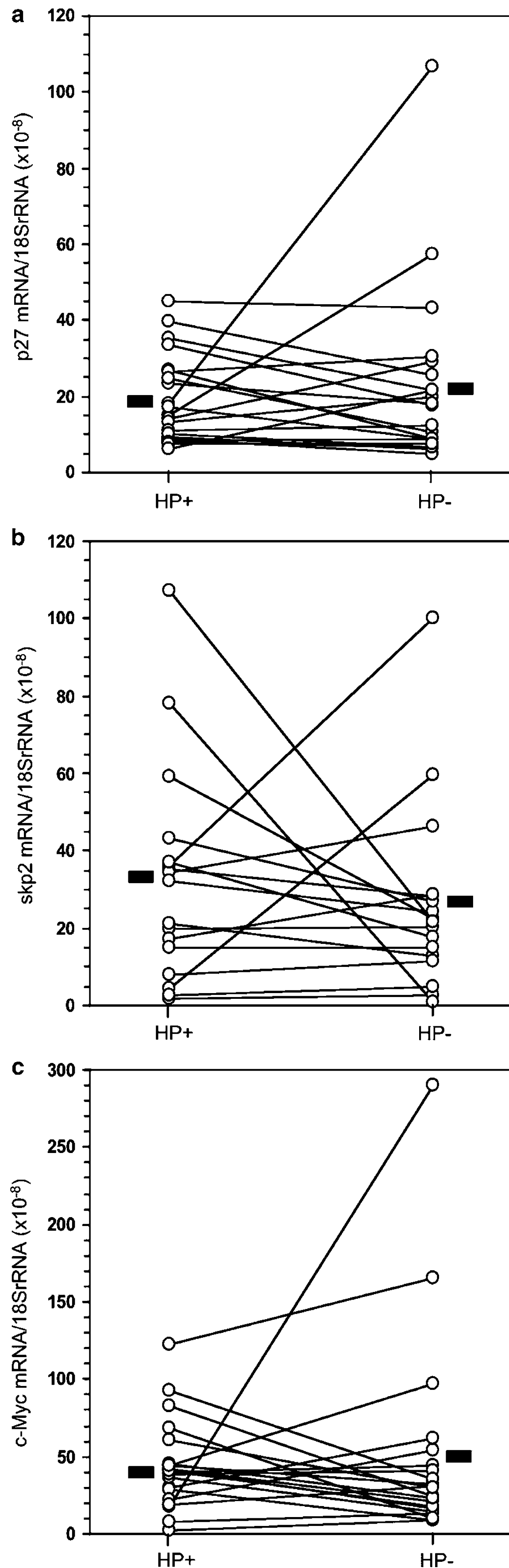


Figure 4 IL-8 mRNA was significantly lower in gastric epithelial cells after the eradication of *H. pylori*. IL-8 mRNA was measured by real-time PCR of laser-captured microdissected RNA and normalized for 18SrRNA. Thick black bars indicates the mean values, $**P<0.001$.

decreasing p27 at multiple levels, including transcriptional regulation and cytoplasmic sequestration of p27, allowing for cell cycle progression from G1 to S phases.[36,37] We did not observe cytoplasmic p27 expression in gastric epithelial cells even before eradication when c-Myc was increased, but this may be due to a relative lack of sensitivity of immunostaining. The relationship between c-Myc, Skp2, and p27 is complex. Skp2 has ubiquitin ligase activity for c-Myc, leading to its ubiquitination and destruction in the proteasome, but Skp2 also can stimulate c-Myc transcription.[30,31] As cells progress to S phase, increasing levels of Skp2 therefore lead not only to the targeting of p27 for proteasomal degradation but also the transient activation of c-Myc. c-Myc then synergizes with the decrease in p27 protein level to enforce the commitment of cells to enter S phase. Thus, the results of this *in vivo* study are supportive of the hypothesis that, in *H. pylori*-associated gastritis, increased Skp2 may be the mechanism common to both downregulation of p27 and increased c-Myc, the net result of which is increased cell proliferation. However, further dissection of the responsible molecular mechanisms will be necessary because of the evidence that Skp2 is not involved in the downregulation of p27 by *H. pylori in vitro*.[28]

Figure 3 Example of LCM. (**a**) The target gastric epithelium was marked digitally, shown here as a red color. (**b**) After laser microdissection, gastric epithelial cells were captured on a Capsure Macro LCM cap. (**c**) Residual tissue after microdissection has removed the gastric epithelial cells of interest.



In this study we used LCM to extract RNA, thus allowing us to examine in parallel the expression of protein and mRNA for *p27*, *skp2*, and *c-myc*. As p27 is highly expressed in inflammatory cells that accumulate in the lamina propria during *H. pylori* infection, LCM allowed us to specifically examine the regulation of p27 in gastric epithelial cells in the absence of contaminating nonepithelial cells. While changes in p27, Skp2, and c-Myc were all highly statistically significant at the protein level, their mRNA expression was not significantly influenced by *H. pylori* eradication. To confirm that this was not an artifact of the LCM itself, we also performed in parallel evaluation of the mRNA expression of *IL-8*, known to be regulated transcriptionally by *H. pylori* in gastric epithelial cells.[44,52] *IL-8* mRNA was significantly lower following the eradication of *H. pylori* confirming these previous reports, providing evidence that the relative stability of p27, Skp 2, and c-Myc transcripts following the eradication of *H. pylori* in gastric epithelial cells is an authentic finding. Apart from its effects on p27, post-translational regulation of epithelial cell gene expression by *H. pylori* has not been previously emphasized. *H. pylori* does not generally upregulate proteasomal activity, as determined by fluorogenic substrate assays,[28] but it may have specific effects on proteasomal function or eukaryotic protein targeting, as has been demonstrated for some other pathogenic or commensal bacteria.[53,54]

A limitation of our study was that relatively few patients were included and only two endoscopic antral biopsies were available at each time point for each patient, thus allowing us to sample only a small percentage of the gastric mucosa. Sampling variability might be responsible for some of the outlying data points evident in mRNA analysis. However, a strength of the study design was the ability to use each patient as their control in paired analysis and the fact that the study population was ethnically relatively homogenous. While the acid-secretory status of the subjects was not determined in this study, it is likely that, because almost all the subjects had histological evidence of intestinal metaplasia or atrophic gastritis, some may have already developed hypochlorhydria with the subsequent overgrowth of non-*Helicobacter* bacteria and gastric nitrosamine generation.[55] Variability in acid-secretory status may have conceivably influenced our results—the effects of acid secretion or nitrosamine generation on the expression of gastric p27, Skp2, or c-Myc are not known.

Figure 5 Changes in mRNA transcript number for (**a**) p27, (B) Skp2, and (**c**) c-Myc in gastric epithelial cells of 22 patients after the eradication of *H. pylori*. RNA was extracted from laser-captured microdissected epithelial cells, measured by real-time PCR, and normalized for 18SrRNA. Thick black bars indicates the mean values. None was significantly changed following *H. pylori* eradication.

In conclusion, *H. pylori* downregulates p27 through increasing p27 protein degradation *in vivo* and this is reversed following *H. pylori* eradication. Our study indicates that the possible pathways mediating this effect may involve both Skp2 and c-Myc. The ability of *H. pylori* to downregulate p27 may provide a mechanistic link between *H. pylori*, gastric epithelial cell hyperproliferation, and gastric carcinogenesis.

Acknowledgements

This work was supported by the US National Institutes of Health (National Institute for Research Resources, 1P20RR17695-01).

References

1 IARC Working Group on the Evaluation of Carcinogenic Risks to Humans. Schistosomes, liver flukes and *Helicobacter pylori*. IARC Monogr Eval Carcinogen Risks Hum 1994;61:1–241.
2 Peek Jr RM, Blaser MJ. *Helicobacter pylori* and gastrointestinal tract adenocarcinomas. Nat Rev Cancer 2002;2:28–37.
3 Pisani P, Parkin DM, Munoz N, *et al.* Cancer and infection: estimates of the attributable fraction in 1990. Cancer Epidemiol Biomarkers Prev 1997;6:387–400.
4 El-Omar EM, Carrington M, Chow WH, *et al.* Interleukin-1 polymorphisms associated with increased risk of gastric cancer. Nature 2000;404:398–402.
5 Blaser MJ, Atherton JC. *Helicobacter pylori* persistence: biology and disease. J Clin Invest 2004;113: 321–333.
6 Lauren P. The two histological main types of gastric carcinoma: diffuse and so-called intestinal-type carcinoma. An attempt at a histo-clinical classification. Acta Pathol Microbiol Scand 1965;64:31–49.
7 Correa P, Haenszel W, Cuello C, *et al.* A model for gastric cancer epidemiology. Lancet 1975;2:58–60.
8 Yuasa Y. Control of gut differentiation and intestinal-type gastric carcinogenesis. Nat Rev Cancer 2003;3: 592–600.
9 Shirin H, Weinstein IB, Moss SF. Effects of *H. pylori* infection of gastric epithelial cells on cell cycle control. Front Biosci 2001;6:E104–E118.
10 Preston-Martin S, Pike MC, Ross RK, *et al.* Increased cell division as a cause of human cancer. Cancer Res 1990;50:7415–7421.
11 Philipp-Staheli J, Payne SR, Kemp CJ. p27(Kip1): regulation and function of a haploinsufficient tumor suppressor and its misregulation in cancer. Exp Cell Res 2001;264:148–168.
12 Fero M, Randel E, Gurley KE, *et al.* The murine gene $p27^{Kip1}$ is haplo-insufficient for tumor suppression. Nature 1998;396:177–180.
13 Besson A, Gurian-West M, Schmidt A, *et al.* $p27^{Kip1}$ modulates cell migration through the regulation of RhoA activation. Genes Dev 2004;18:862–876.
14 Nakayama K, Nagahama H, Minamishima YA, *et al.* Skp2-mediated degradation of p27 regulates progression into mitosis. Dev Cell 2004;6:661–672.
15 Pagano M, Tam SW, Theodoras AM, *et al.* Role of the ubiquitin–proteasome pathway in regulating abundance of the cyclin-dependent kinase inhibitor p27. Science 1995;269:682–685.
16 Roos-Mattjus P, Sistonen L. The ubiquitin–proteasome pathway. Ann Med 2004;36:285–295.
17 Carrano AC, Eytan E, Hershko A, *et al.* Skp2 is required for ubiquitin-mediated degradation of the CDK inhibitor p27. Nat Cell Biol 1999;1:193–199.
18 Tsvetkov LM, Yeh KH, Lee SJ, *et al.* $p27^{Kip1}$ Ubiquitination and degradation is regulated by the SCFSkp2 complex through phosphorylated Thr187 in p27. Curr Biol 1999;9:661–664.
19 Nakayama K-I, Hatakeyama S, Nakayama K. Regulation of the cell cycle at the G1–S transition by proteolysis of cyclin E and $p27^{Kip1}$. Biochem Biophys Res Commun 2001;282:853–860.
20 Nakayama K, Nagahama H, Minamishima YA, *et al.* Targeted disruption of Skp2 results in accumulation of cyclin E and $p27^{Kip1}$, polyploidy, and centrosome overduplication. EMBO J 2000;19:2069–2081.
21 Hara T, Kamura T, Nakayama K, *et al.* Degradation of p27(Kip1) at the G(0)–G(1) transition mediated by a Skp2-independent ubiquitination pathway. J Biol Chem 2001;276:48937–48943.
22 Mori M, Mimori K, Shiraishi T, *et al.* p27 expression and gastric carcinoma. Nat Med 1997;3:593.
23 Ohtani M, Isozaki H, Fujii K, *et al.* Impact of the expression of cyclin-dependent kinase inhibitor $p27^{Kip1}$ and apoptosis in tumor cells on the overall survival of patients with non-early stage gastric carcinoma. Cancer 1999;85:1711–1718.
24 Tahara E. Genetic pathways of two types of gastric cancer. IARC Sci Publ 2004;157:327–349.
25 Sgambato A, Migaldi M, Leocata P, *et al.* Loss of $p27^{Kip1}$ expression is a strong independent prognostic factor of reduced survival in N0 gastric carcinomas. Cancer 2000;89:2247–2257.
26 Shirin H, Sordillo EM, Kolevska TK, *et al.* Chronic *Helicobacter pylori* infection induces an apoptosis-resistant phenotype associated with decreased expression of p27(kip1). Infect Immun 2000;68:5321–5328.
27 Yu J, Leung WK, Ng EK, *et al.* Effect of *Helicobacter pylori* eradication on expression of cyclin D2 and p27 in gastric intestinal metaplasia. Aliment Pharmacol Ther 2001;15:1505–1511.
28 Eguchi H, Herschenhous N, Kuzushita N, *et al.* *Helicobacter pylori* increases proteasome-mediated degradation of p27(kip1) in gastric epithelial cells. Cancer Res 2003;63:4739–4746.
29 Masuda TA, Inoue H, Sonoda H, *et al.* Clinical and biological significance of S-phase kinase-associated protein 2 (Skp2) gene expression in gastric carcinoma: modulation of malignant phenotype by Skp2 overexpression, possibly via p27 proteolysis. Cancer Res 2002;62:3819–3825, 26.
30 Kim SY, Herbst A, Tworkowski KA, *et al.* Skp2 regulates Myc protein stability and activity. Mol Cell 2003;11:1177–1188.
31 von der Lehr N, Johansson S, Wu S, *et al.* The F-box protein Skp2 participates in c-Myc proteasomal degradation and acts as a cofactor for c-Myc-regulated transcription. Mol Cell 2003;11:1189–1200.
32 Kamura T, Hara T, Kotoshiba S, *et al.* Degradation of $p57^{Kip2}$ mediated by SCFSkp2-dependent ubiquitylation. Proc Natl Acad Sci USA 2003;100:10231–10236.

33 Liang M, Liang YY, Wrighton K, *et al.* Ubiquitination and proteolysis of cancer-derived Smad4 mutants by SCFSkp2. Mol Cell Biol 2004;24:7524–7537.
34 Marti A, Wirbelauer C, Scheffner M, *et al.* Interaction between ubiquitin-protein ligase SCFSKP2 and E2F-1 underlies the regulation of E2F-1 degradation. Nat Cell Biol 1999;1:14–19.
35 Grandori C, Cowley SM, James LP, *et al.* The Myc/Max/Mad network and the transcriptional control of cell behavior. Annu Rev Cell Dev Biol 2000;16:653–699.
36 Yang W, Shen J, Wu M, *et al.* Repression of transcription of the p27(Kip1) cyclin-dependent kinase inhibitor gene by c-Myc. Oncogene 2001;20:1688–1702.
37 Perez-Roger I, Kim SH, Griffiths B, *et al.* Cyclins D1 and D2 mediate myc-induced proliferation via sequestration of p27(Kip1) and p21(Cip1). EMBO J 1999;18: 5310–5320.
38 Suerbaum S, Michetti P. *Helicobacter pylori* infection. N Engl J Med 2002;347:1175–1186.
39 Sgambato A, Zhang YJ, Ciaparrone M, *et al.* Overexpression of $p27^{Kip1}$ inhibits the growth of both normal and transformed human mammary epithelial cells. Cancer Res 1998;58:3448–3454.
40 Mateyak MK, Obaya AJ, Adachi S, *et al.* Phenotypes of c-Myc-deficient rat fibroblasts isolated by targeted homologous recombination. Cell Growth Differ 1997; 8:1039–1048.
41 Eguchi H, Carpentier S, Kim SS, *et al.* $P27^{kip1}$ regulates the apoptotic response of gastric epithelial cells to *Helicobacter pylori*. Gut 2004;53:797–804.
42 Dixon MF, Genta RM, Yardley JH, *et al.* Classification and grading of gastritis. The updated Sydney System. International Workshop on the Histopathology of Gastritis, Houston 1994. Am J Surg Pathol 1996;20: 1161–1181.
43 Moss SF, Legon S, Davies J, *et al.* Cytokine gene expression in *Helicobacter pylori* associated antral gastritis. Gut 1994;35:1567–1570.
44 Crabtree JE. Immune and inflammatory responses to *Helicobacter pylori* infection. Scand J Gastroenterol 1996;215(Suppl):3–10.
45 Sougioultzis S, Foukas PG, Tzivras M, *et al.* Alterations in the proliferating compartment of gastric mucosa during *Helicobacter pylori* infection: the putative role of epithelial cells expressing p27(kip1). Mod Pathol 2003;16:1076–1085.
46 Wirbelauer C, Sutterluty H, Blondel M, *et al.* The F-box protein Skp2 is a ubiquitylation target of a Cul1-based core ubiquitin ligase complex: evidence for a role of Cul1 in the suppression of Skp2 expression in quiescent fibroblasts. EMBO J 2000;19:5362–5375.
47 Bashir T, Dorrello NV, Amador V, *et al.* Control of the SCF(Skp2-Cks1) ubiquitin ligase by the APC/C(Cdh1) ubiquitin ligase. Nature 2004;428:190–193.
48 Wei W, Ayad NG, Wan Y, *et al.* Degradation of the SCF component Skp2 in cell-cycle phase G1 by the anaphase-promoting complex. Nature 2004;428: 194–198.
49 Shirin H, Sordillo EM, Oh SH, *et al.* *Helicobacter pylori* inhibits the G1 to S transition in AGS gastric epithelial cells. Cancer Res 1999;59:2277–2281.
50 Nardone G, Staibano S, Rocco A, *et al.* Effect of *Helicobacter pylori* infection and its eradication on cell proliferation, DNA status, and oncogene expression in patients with chronic gastritis. Gut 1999;44: 789–799.
51 Nardone G, Staibano S, Rocco A, *et al.* Effect of *Helicobacter pylori* infection on gastric cell proliferation and genomic instability in a paediatric population of southern Italy. Digest Liver Dis 2001; 33:743–749.
52 Sharma SA, Tummuru MK, Miller GG, *et al.* Interleukin-8 response of gastric epithelial cell lines to *Helicobacter pylori* stimulation *in vitro*. Infect Immun 1995;63:1681–1687.
53 Boyer L, Lemichez E. Targeting of host-cell ubiquitin and ubiquitin-like pathways by bacterial factors. Nat Rev Microbiol 2004;2:779–788.
54 Petrof EO, Kojima K, Ropeleski MJ, *et al.* Probiotics inhibit nuclear factor-kappaB and induce heat shock proteins in colonic epithelial cells through proteasome inhibition. Gastroenterology 2004;127:1474–1487.
55 Iijima K, Sekine H, Koike T, *et al.* Long-term effect of *Helicobacter pylori* eradication on the reversibility of acid secretion in profound hypochlorhydria. Aliment Pharmacol Ther 2004;19:1181–1188.