microRNA profiling in duodenal ulcer disease caused by *Helicobacter pylori* infection in a Western population

S. Lario1,2,*, M. J. Ramírez-Lazáro1,2,*, A. M. Aransay3, J. J. Lozano4, A. Montserrat1,2, Á. Casalots5, F. Junquera1,2, J. Álvarez3, F. Segura1, R. Campo1,2 and X. Calvet1,2

1) Departament de Medicina, Digestive Diseases Service, Hospital de Sabadell, Institut Universitari Parc Taulí, Universitat Autònoma de Barcelona, Sabadell, Barcelona, Spain, 2) Centro de Investigación Biomédica en Red de Enfermedades Hepáticas y Digestivas (CIBEREHD), Instituto de Salud Carlos III, 3) Genome Analysis Platform, CIC bioGUNE, 4) Plataforma de Bioinformática, Centro de Investigación Biomédica en Red de Enfermedades Hepáticas y Digestivas (CIBEREHD), Instituto de Salud Carlos III, 5) Departament de Medicina, Servei de Patologia, UDIAT-CD, Hospital de Sabadell, Institut Universitari Parc Taulí, Universitat Autònoma de Barcelona and 6) Departament de Medicina, Servei de Malalties Infeccioses, Hospital de Sabadell, Institut Universitari Parc Taulí, Universitat Autònoma de Barcelona, Spanish Network for the Research in Infectious Diseases (REIPI RD06/0018), Sabadell, Barcelona, Spain

Abstract

Although the connection of microRNAs (miRNAs) to some diseases is well established, their involvement in chronic infections such as *Helicobacter pylori* has received less attention. The aim was to compare miRNA expression profiling in patients with duodenal ulcer (DU) due to *H. pylori* infection with that in infected patients without DU and in uninfected patients. The miRNA expression profile was determined by microarrays in antral mucosal samples from well-characterized dyspeptic patients (*n* = 46). The most significant set of miRNAs was subsequently analysed in an independent validation group of patients (*n* = 42). Transcripts for IL8, IL12p40, IL12p35 and IL23p19, the signalling molecules MYD88, GATA6, SOCS2 and STAT6 and *H. pylori* virulence factors cagA and VacA were analysed. Microarray experiments showed that 17 miRNAs were deregulated in the mucosa of *H. pylori*-infected patients. No significant differences were observed between normal and DU patients. PCR confirmed the up-regulation of miR-9, miR-146a, miR-155 and miR-650 and the down-regulation of miR-96 and miR-204 in the independent validation set of patients. Importantly, miR-9, miR-96, miR-146a and miR-650 expression was specific to chronic-active gastritis. *H. pylori*-infected patients showed higher levels of IL8 and IL12p40 mRNAs and lower levels of GATA6 and SOCS2 mRNAs. The antral mucosa of patients with non-active or chronic-active gastritis showed significantly lower levels of GATA6, MYD88, SOCS2 and STAT6 mRNAs compared with patients without gastritis. The down-regulation of these factors was not correlated with the expression of any of the validated miRNAs. The exact role of the miRNA changes observed will require further study.

Keywords: Chronic active gastritis, duodenal ulcer disease, *Helicobacter pylori*, microRNA

Original Submission: 7 October 2011; Revised Submission: 5 January 2012; Accepted: 8 March 2012

Editor: F. Megraud

Clin Microbiol Infect

**Corresponding author:** X. Calvet, MD, PhD, Unitat de Malalties Digestives, Hospital de Sabadell, Institut Universitari Parc Taulí, Universitat Autònoma de Barcelona, CIBEREHD – Instituto de Salud Carlos III, Parc Taulí, s/n, 08208 Sabadell (Barcelona), Spain

E-mail: xcalvet@tauli.cat

*These authors contributed equally to the work.

Introduction

*Helicobacter pylori* has infected human beings for the past 58 000 years [1]. In most infected people, the bacterium acts as a commensal organism inducing asymptomatic chronic active gastritis that can last for life. In other cases, however, it is responsible for a heavy toll of morbidity and mortality as a consequence of peptic ulcers and gastric cancer.

It is a well-known fact that *H. pylori*-infected patients with duodenal ulcer disease are at a lower risk of developing gastric cancer than patients with gastric ulcerations [2]. Chronic active gastritis in duodenal ulcer disease does not usually lead to preneoplastic changes such as atrophy or metaplasia, as it does in patients with gastric ulcer or non-ulcer dyspepsia. This wide individual response to infection has been
attributed to environmental, host and bacterial virulence factors [3]. Despite intensive research in this field, clear risk or disease-specific markers remain elusive.

Until recently, most of the known non-coding RNAs (ncRNAs) have been implicated in housekeeping functions in cells [4]. But in the past few years, thousands of genes that produce ncRNA transcripts with no recognizable open reading frame have been discovered [4,5]. MicroRNAs (miRNAs) have received considerable attention because of their impact on transcription output. miRNAs are a family of ncRNAs involved in key biological processes such as differentiation, development and cell growth [6].

miRNAs were first discovered in genetic screens of Caenorhabditis elegans, and play a relevant role in the development and growth of the worm [7]. This seminal discovery led to the identification of the orthologous miRNA let-7 in human cancer. Since then, numerous studies have focused on the profiling of miRNAs in this setting [8]. Studies that analyse gastric cancer have shown an altered profile of expression (reviewed in [9,10]). Although the connection of miRNAs to some diseases is well established, their involvement in the response to bacterial infectious diseases has received much less attention. In fact, only two studies have determined the miRNA expression profile in the gastric mucosa of non-cancer patients infected with H. pylori [11,12]. Both studies were performed in Asian populations and showed the deregulation of miRNAs in H. pylori-infected patients.

The aim of this study was to identify and assess the microRNA expression pattern in H. pylori-infected patients with duodenal ulcer disease.

**Patients and Methods**

Additional information about the reference standard, H. pylori genotyping, PCR primers and molecular analyses can be found in Supporting Information.

**Patients**

Outpatients sent to the Hospital de Sabadell for dyspeptic symptoms from October 2006 to March 2010 were prospectively recruited. Patients were contacted prior to the endoscopy and were asked to participate. Before the endoscopy, the patient signed the informed consent document in accordance with the policy and guidelines of the Ethical Committee of our institution. Those who accepted were instructed to avoid antisecretory drugs in the 2 weeks before the test. Patients unable to stop antisecretory drugs, those who had received antibiotics in the 4 weeks before the endoscopy and those with previous H. pylori treatment were excluded. During endoscopy, antral biopsies were obtained for histology, rapid urease test (RUT) and molecular studies. As gastric biopsy specimens can show different levels of metaplasia or atrophy and microRNA profiling is capable of identifying cell lineages and tissues [13,14], patients with metaplasia or atrophy were excluded in order to avoid potential confounding variables. Reference standard-negative patients with lymphoid aggregates were also excluded.

The study design is depicted in Fig. 1 and patients’ clinical and demographic data are shown in Table 1.

**Reference standard**

Stringent patient selection criteria were used. A patient was classified as being H. pylori positive if the results of the UBT, RUT, H. pylori culture and histology were all positive. Patients were defined as H. pylori negative if all tests were negative.

**H. pylori genotyping**

PCR was used to determine H. pylori cagA status and to genotype the vacA gene (signal s1/s2, intermediate i1/i2 and midregion m1/m2 polymorphisms) as described previously (detailed information on primer sequences and PCR conditions is presented in Table S1). Isolates were classified as high virulence (cagA+ VacA s1m1i1), low virulence (cagA- VacA s2m2i2) or mixed.

**High-throughput miRNA microarrays**

miRNA profiling was performed with Human v1 MicroRNA expression beadchips (Illumina Inc., San Diego, CA, USA).

FIG. 1. Study flow diagram.
Reverse transcription and real-time PCR
Transcripts for interleukins IL8, IL23p19, IL12p35 and IL12p40 and selected signalling molecules GATA6, MYD88, SOCS2 and STAT6 were investigated (Table S2).

miR-9, miR-96, miR-98, miR-137, miR-146a, miR-155, miR-196a, miR-204, miR-519e and miR-650 expression was assessed by miRCURY LNA™ Universal RT microRNA PCR system (Exiqon, Vedbaek, Denmark) (Table S3).

Data and statistical analysis
The Illumina Beadchip raw data were imported to BeadStudio v3.2 (Illumina Inc.) and subsequently analysed as detailed in Supplementary Methods. LinRegPCR v12.0 was used to calculate the starting concentration per sample (No) [15]. Detailed information is provided in Supporting Information.

Differences between groups were analysed using the Mann–Whitney U-test or the Kruskal–Wallis H-test as appropriate. Correlation was assessed by Spearman’s rho. ROC curves were used to examine the diagnostic accuracy of miRNAs. Statistical analyses were performed with SPSS v19 (IBM Corporation, Armonk, NY, USA). Significance was set at p <0.05.

Results

H. pylori infection alters miRNA expression in the antral mucosa
Seventeen miRNAs were deregulated in the mucosa of H. pylori-infected patients (p <0.05) (Table 2 and Fig S1). miR-96 was a 1.5-fold down-regulated in patients with gastritis (p 0.0144). Globaltest analyses (Table S4) showed no significant differences with respect to sex, age, endoscopic diagnosis (normal vs. duodenal ulcer) or H. pylori virulence factors (high virulence (cagA+ VacA s1m1i1) vs. low virulence (cagA- VacA s2m2i2)).

Microarray data have been deposited in NCBI’s Gene Expression Omnibus [16] (GEO Series accession number GSE32174).

The expression of the selected miRNAs is confirmed in the validation group of dyspeptic patients
miRNA RT-qPCR experiments performed on antral biopsies from the independent validation group of dyspeptic patients showed no statistical differences between normal and duodenal ulcer patients (Fig. 2). However, the increased expression of miR-9, miR-146a, miR-155 and miR-650 (Fig. 3a) and the down-regulation of miR-96 and miR-204 (Fig. 3b) was confirmed.

The highest fold increases in miRNA expression related to chronic active gastritis were for miR-650 (c. 10-fold), followed by miR-146a (c. 6-fold) and miR-155 (c. 3-fold). miR-9,
miR-204 and miR-96 fold-changes in chronic active gastritis vs. non-infected patients were 1.9, −2.0 and −2.4, respectively.

Non-active gastritis in the antrum is a common histological diagnosis in dyspeptic patients. In our consecutive cohort of dyspeptic patients (n = 425), 175 patients (41%) were H. pylori negative. Among the H. pylori-negative cohort, 77% of biopsies showed mild non-active gastritis according to the pathologist’s report (unpublished observations). As non-active gastritis is very common and this group was not analysed in the microarray experiments, we decided to analyse a group of 13 additional patients who were H. pylori negative with non-active gastritis. RT-qPCR experiments showed no differences in miRNA expression between H. pylori-negative patients with and without non-active gastritis (Fig. 3a,b).
Correlations for the validated miRNAs in relation to reference standard and gastritis activity

miR-146a and miR-155 expressions were highly correlated (Fig. 4) and independent of H. pylori status (reference negative without gastritis, $r_s = 0.9000 \ p <0.01$) or gastritis activity (non-active gastritis, $r_s = 0.7032, \ p <0.01$; chronic active gastritis, $r_s = 0.7108, \ p <0.01$). Interesting results were also found for the pair miR-155 and miR-204, which were correlated in infected but not in non-infected patients ($r_s = 0.6544, \ p <0.01$) and in patients with active but not in patients with non-active gastritis. miR-650 was correlated with miR-146a and miR-155 in non-infected patients (miR-650 vs. miR-146a, $r_s = 0.8786, \ p <0.01$; miR-650 vs. miR-155, $r_s = 0.8284, \ p <0.01$). Table S5 summarizes the coefficients of correlations for all validated miRNAs in relation to gastritis activity.
IL8, IL12p40, IL12p35 and IL23p19 mRNA expression

We decided to determine IL8 expression because two of the validated miRNAs from this study (miR-155 and miR-146a) have been previously involved in its regulation [17–20]. Additionally, miR-155 also modulates the expression of IL-23 [21]. IL-23 is a heterodimer formed by IL23p19 and IL12p40 subunits. The IL12p40 subunit is also shared to form the cytokine IL-12 by combining with IL12p35.

Antral biopsies from H. pylori-infected patients showed increased IL8 and IL12p40 mRNA expression (reference negative vs. positive: IL8, p <0.00001; IL12p40, p <0.0001) (Fig. S3), which was significantly associated with gastritis activity (non-active vs. chronic active gastritis: IL8, p <0.0001; IL12p40, p 0.0018) (Fig. 5a). Patients with duodenal ulcer or erosive duodenitis had a higher expression of IL8 mRNA than infected non-ulcer subjects (p 0.0076).

In vivo IL8, IL12p40, IL12p35 and IL23p19 expression was not correlated with the validated miRNAs (Table S6).

Expression of the signalling molecules MYD88, GATA6, SOCS2 and STAT6

We determined MYD88 mRNA expression in our group of H. pylori-infected patients because miR-9, miR-155 and miR-146a are induced by Toll-like receptor-2 (TLR-2) and TLR-2 signals through MYD88 [18]. MYD88 mRNA expression was not deregulated in H. pylori-infected patients (Fig. S3), and miR-9, miR-155 and miR-146a expression was not correlated with MYD88 expression (Table S6).

Previous microarray and immunochemistry experiments [22] have revealed the mRNA and protein down-regulation of the transcription factors GATA6 and STAT6 and the suppressor of cytokine signaling-2 (SOCS2) in the antral mucosa of H. pylori-infected patients. We therefore sought to correlate these findings with our miRNA signature. Our cohort of patients confirms that the expression of GATA6 and SOCS2 is down-regulated in H. pylori-positive patients (reference negative vs. positive: GATA6, p 0.0363; SOCS2, p 0.0060) (Fig. S3). None of these genes, however, was associated with gastritis activity (Fig. 5b) or the presence of duodenal disease (not shown). GATA6, STAT6 or SOCS2 levels were not correlated with the validated miRNAs (Table S6).

H. pylori virulence factors were associated with IL8 and IL12p40 mRNA levels but not with validated miRNAs

CagA was present in 14 (70%) out of the 20 H. pylori isolates. IL8 and IL12p40 mRNA levels were higher in the antral biopsies of patients infected with cagA+ strains (IL8, p 0.0340; IL12p40, p 0.0262) (Fig. S4). Confirming our microarray results, miR-9, miR-96, miR-98, miR-146a, miR-155, miR-204 and miR-650 expression in the H. pylori-infected patients was

FIG. 4. Correlation plots for the selected miRNAs in relation to gastritis activity. \( r_s \), Spearman’s correlation coefficient.
FIG. 5. mRNA gene expression of selected interleukins (a), signalling molecules (b) and the housekeeping gene (c) in relation to gastritis activity. Kruskal-Wallis H-test. Box plots show the 25th, 50th (median), 75th and range (whiskers).
independent of the presence of cagA or vacA genotype (Table S7).

The ratio miR-96/miR-155 shows the best diagnostic performance
Among the individual miRNAs, only miR-650 showed high performance with an AUC of 0.8659 (p <0.0001) (Table S8). Sensitivities and specificities were improved when the ratio between down-regulated (miR-204 and miR-96) and up-regulated (miR-9, miR-146a, miR-155 and miR-650) miRNAs was used. Among these, the ratio miR-96/miR-155 was the best with AUC = 0.9665 (p <0.0001) with a sensitivity of 84.2% and a specificity of 95.5%.

Discussion
The present study was designed to analyse miRNA expression in human H. pylori-infected patients with or without duodenal ulcer disease, but with no histological diagnosis of metaplasia or atrophy. This strict patient selection criterion simplified microarray data analysis and interpretation, because miRNA signatures are characteristic of cell lineages and tissues [13]. Microarray results showed that miRNAs are differentially expressed in the H. pylori-infected antrum. In particular, 17 miRNAs were up-regulated and three were down-regulated in the mucosa of H. pylori-infected patients. The altered miRNA expression was not significantly associated with duodenal ulcer disease or with the presence of H. pylori virulence factors cagA or vacA. We could confirm the up-regulation of miR-9, miR-146a, miR-155 and miR-650 and the down-regulation of miR-96 and miR-204 in the independent set of dyspeptic patients.

Thus far, only one report has described the miRNA profile of H. pylori infection in non-cancer patients [11]. Our miRNA signature is, however, significantly different. The only factor shared by both studies is the down-regulation of miR-204. Several explanations can be proposed for this discrepancy. First, the population studied: the previous study was performed in Japanese patients, whereas the present study comes from a Western population. Apart from the different genetic background of the populations analysed, it is widely accepted that H. pylori East Asian strains are more virulent than Western ones [23]. Second, our study reports the miRNA profiling of infected patients with strict criteria for assuming the absence of infection and the presence of normal mucosa (i.e. without metaplasia or atrophy). Finally, there are some methodological differences in the microarray platforms used [24]. A major one is the preamplification step performed prior to hybridization in our study. Nevertheless, both studies were able to confirm their respective microarray results by RT-qPCR in a validation set of patients.

TLRs play an important role in maintaining mucosal homeostasis [25]. After pathogen recognition, TLRs signal through the adaptor molecule MYD88, which activates downstream factors to coordinate the up-regulation of several functionally distinct gene subsets. Moreover, MYD88-deficient dendritic cells show impaired ability to mount local gastric inflammatory responses after H. pylori infection [26]. miR-9, miR-146a and miR-155 are miRNAs regulated by TLR in different cell types and some participate in the fine-tune control of TLR signalling components [18]. miR-155 is induced in cultured human T cells infected with H. pylori or treated with the purified H. pylori components lipopolysaccharide, VacA and γ-glutamyl transeptidase through the activation of the transcription factor Foxp3 and cAMP [27]. We analysed MYD88 mRNA expression, and found no association with H. pylori infection. This effect may be explained by the recent observation that although MYD88 is a target of miR-155, it is repressed by translational repression and not by mRNA cleavage [20]. Further investigations will determine whether this apparent discrepancy reflects a different regulation of MYD88 by miR-155 in vivo (gastric mucosa) or in vitro (cultured gastric cells). The same study reported in vitro inhibition of IL8 by miR-155. In macrophages, miR-155 is a common target of a broad range of inflammatory mediators including MYD88 and TRIF through TLR signalling and TNF-α [28]. Additionally, miR-155 shows oncogenic properties [29]. The role of miR-155 in the induction and maintenance of chronic active gastritis provoked by H. pylori and its progression to intestinal metaplasia, dysplasia and eventually gastric cancer will be studied in the future.

Experiments performed with an acute monocytic leukaemia cell line stimulated with LPS have revealed that miR-146a expression is induced by the NF-κB cascade activated after the pathogen component has been recognized by the TLR system [30]. Gastric epithelial cells infected with H. pylori have shown that induced miR-146a down-regulates IRAK1 and TRAF6 proteins, participating in a negative loop that restrains the excessive activity of the pathway [31]. Others, by analysing epithelial cells and neutrophils isolated from cystic fibrosis patients, have found that miR-146a is also implicated in the regulation of IL8 [17]. IL-8 is a potent neutrophil-activating chemokine released by gastric epithelial cells infected with H. pylori [3]. The H. pylori-infected antral biopsies from the present study showed increased IL8 and IL12p40 mRNA expression. However, the set of validated miRNAs did not correlate with IL8 or IL12p40 expression. The cellular heterogeneity within antral biopsies may help explain the lack of in vivo correlation between IL8 and
miR-155 or miR-146a expression, because miRNA expression is tightly regulated within cellular populations [13,14].

More striking was the up-regulation of miR-650 in our dataset. To the best of our knowledge, miR-650 expression has not been associated with epithelial physiology or immunological response. The normal antral mucosa expressed miR-650 at low levels and it was induced in infected patients with chronic active gastritis. Very little is known about this miRNA: it has been implicated in the development of distant metastasis in gastric cancer patients [32] and is induced in tumoural samples from colorectal cancer patients [33].

Previous microarray and immunohistochemistry experiments [22] have demonstrated the mRNA and protein down-regulation of GATA6, STAT6 and SOCS2 in the antral mucosa of H. pylori-infected patients. Our results confirm the down-regulation of GATA6 and SOCS2 expression in H. pylori infection but we could not find a relationship between the validated miRNAs and the mRNA levels of these genes.

H. pylori strains harbouring multiple virulence factors (cagA+, VacA s1m1i1) are associated with increased gastric mucosal inflammatory cell infiltration and increased gastric epithelial injury [34]. Accordingly, our results show the up-regulation of IL8 and IL12p40 in the antral biopsies of patients infected with cagA+ strains but we were unable to associate virulence factors with other variables. These results might be limited by sample size; therefore, additional studies will be required.

Finally, we examined the possibility of using the validated miRNAs as novel surrogate markers for H. pylori infection. The individual miRNA diagnostic performance was suboptimal, but by calculating the ratios between down-regulated and up-regulated miRNAs the sensitivities and specificities improved significantly, with sensitivities of c. 85% and specificities of c. 95%. The prospect that miRNAs might be used as novel surrogate markers for H. pylori infection opens up new possibilities for the early diagnosis of H. pylori infection, especially in cases in which H. pylori detection is difficult, for instance in patients with bleeding peptic ulcers [35].

The miRNA regulation reported by our study generates a tentative miRNA signature for H. pylori infection, but in order to obtain a definitive set of markers, we need to identify new, currently unknown miRNAs and demonstrate their association with infectious diseases. Microarrays are high-throughput and highly reproducible, but rely on the existing knowledge of the sequences to be studied. Therefore, high-throughput sequencing should be used to obtain complete descriptions of tissue micro-transcriptome regulation.

In conclusion, our results show that a set of miRNAs are deregulated during chronic gastric inflammation and that this set may be useful as a surrogate marker for determining the presence of H. pylori. The exact role of the miRNA changes observed with the development of H. pylori complications and, specifically, with the appearance of gastric neoplasms will require further study.

Acknowledgements

Preliminary study findings were presented at the 23rd (Rotterdam, September 2010) and 24th (Dublin, September 2011) International Workshops on ‘Helicobacter and related bacteria in chronic digestive inflammation and gastric cancer’, organized by the European Helicobacter Study Group (EHSG). We thank Neus Mateo, Laura Moreno and Alicia Sánchez-Rubio for their help in collecting samples and performing the breath test. We are also indebted to Michael Maudsley for his help with the English.

Funding

This study was supported by grants from the Fundació Parc Taulí of the Hospital de Sabadell (CIR2006024, CIR 2008030, CIR 2009054). María-José Ramírez-Lázaro and Sergio Lario are CIBERehd staff researchers. CIBERehd is funded by the Instituto de Salud Carlos III. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Transparency Declaration

The authors declare that no competing interests exist.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Unsupervised hierarchical clustering.
Figure S2. Expression profile of the selected miRNAs in the validation set of dyspeptic patients according to the reference standard for H. pylori infection.
Figure S3. Expression profile of the selected mRNAs in the validation set of dyspeptic patients according to the reference standard for H. pylori infection.
Figure S4. Gene expression of selected interleukins in relation to cagA status.
Table S1. Primers used for H. pylori genotyping.
Table S2. Primers used for RT-qPCR.
Table S3. miRNAs analyzed by RT-qPCR.
Table S4. Results of globaltest analysis.
Table S5. Spearman’s correlation coefficients of miRNA expression.
Table S6. Correlation between miRNAs and interleukins or signaling molecules in relation to *H. pylori* status and gastritis activity.
Table S7. Expression of validated miRNAs in relation to *cagA* status and *vacA* genotypes.
Table S8. Diagnostic accuracy of IL-8 mRNA levels and selected miRNAs.

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.

References