Recent possibilities of detection of Helicobacter pylori infection from biopsy tissue samples in chronic gastritis diseases

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PhD thesis

2004, Budapest
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Introduction

The close correlation between gastritis and peptic ulcer is well documented. Based on the epidemiological findings, these diseases are microbic in the overwhelming majority of chronic gastritis cases, which develop in consequence of the inflammatory reaction against Helicobacter pylori (H.pylori) infection.

The mechanism of the ulcerogenic action of this bacterium and the interference of H.pylori with ulcer healing are yet unknown.

Regulation of cell proliferation and apoptotic process plays a prominent role in maintaining mucosal integrity of gastrointestinal tract. Attention has been recently adverted to the knowledge of H.pylori genotypes by the fact, that its association with role in outcome of cell damage and change of cell kinetic parameters of mucosa. Investigation of different genotypes of H.pylori can support the recognition of part of bacterial factors in the development of gastrointestinal disorders and complications.

No simple quantitative diagnostic test has been validated to determine H.pylori genotypes from fresh and paraffin embedded biopsy samples. Comparison genotypes with histology, endoscopical diagnosis, and non DNA-based techniques (immunohistochemistry, culture, urea breath test, immunoblot) and quantitative evaluation of H.pylori has not happened yet, neither in diagnostic work nor in scientific research.

Moreover, there is no consensus as to whether H.pylori infection in children is more or less severe than in adults.

Objective

The aim of our study was

1. to establish a polymerase chain reaction (PCR) method of H.pylori detection from fresh and paraffin embedded biopsy tissue samples.
2. to develop a new, in situ qualitative-quantitative detection and typing method of H.pylori by real-time PCR assay.
3. to investigate the correlation among our new method and other no-DNA-based diagnostic techniques to detect H.pylori (immunohistochemistry, culture, urea breath test, immunoblot).
4. to investigate the activity of cell proliferation in the presence of H.pylori and different genotypes of bacterium in diversity of histologic stages (normal epithelium, inflammation, ulceration, atrophy, intestinal metaplasia).
5. to investigate the relationship between genotypes of H. pylori and histologic or macroscopic diagnosis.
6. to determine the influence of the presence of H. pylori, its genotypes and the development of intestinal metaplasia on cell proliferation and epidermal growth factor receptor (EGFR) expression.
7. to determine the effect of H. pylori genotypes on proliferation, apoptosis, p53 tumor suppressor gene and intestinal mucin antigens (SIMA, LIMA) expression from child and adult tissue specimens.

**Materials and methods**

During our clinical research we prepared data of 232 patients (116 men and 116 women) according to different respect. We compared results of adult patients with 40 children’s data (17 boys, 23 girls of age averaging at 12.6 ±0.56 years within a range of 4-12 years) used same examination. Children with symptoms of abdominal pain, vomiting and weight loss underwent gastroscopy with biopsy.

32 normal gastric epithelium samples (fresh frozen and paraffin embedded) were used as controls, as well as of small and large intestine biopsy specimens (5 and 5 respectively) for detection of intestinal mucin antigens expression. In child group 5 paraffin embedded normal gastric mucosa samples were used as controls.

Cases were classified according to the macroscopic findings: normal epithelium, endoscopic gastritis, erosion and ulceration. Adult samples were divided according to the routine histology: normal epithelium (n=32), chronic gastritis (n=115), chronic gastritis with intestinal metaplasia (n=75). The specimens were classified in accordance with the Sydney classification.

Bacterial DNA was discovered by lysosime enzyme during the DNA isolation. After DNA isolation conventional PCR method were used for the evaluation of H. pylori cagA, vacA, ureA and iceA genotypes. Then qualitative determination of H. pylori genotypes was made using real-time PCR with SYBR Green dye. The quantitative PCR is based on comparison of expression level of H. pylori ureA and the endogenous expressed ß-globin by FRET (fluorescence resonance energy transfer) method.

The cell kinetics parameters were characterized by immunohistochemistry. Proliferation was assessed by proliferating cell nuclear antigen (PCNA), whereas apoptosis was determined by triphosphate nick-end labelling (TUNEL) immunostaining. Immunohistochemical methods
were used to detect p53 oncoprotein and epidermal growth factor receptor (EGFR) expression, as well as the expression of small and large intestinal mucin antigens (SIMA, LIMA). Other H. pylori diagnostic methods (urea breath test /UBT/, immunoblot) were also used in our study.

Results

1. **H. pylori detection by conventional PCR method:** detection of cagA and vacA genes were amplified from fresh and paraffin embedded biopsy samples, whereas the ureA and iceA gene were detected only from fresh frozen specimens.

2. **Qualitative H. pylori detection by real-time PCR SYBR Green method:** comparison with histology, PCNA and EGFR expression immunostaining of samples. The expression of EGFR was decreased in the presence of cagA and vacA genes.

3. **Quantitative H. pylori detection by real-time PRC FRET method:** comparison with results of histology, urea breath test (UBT), SYBR Green PCR and immunoblot. The real-time PCR FRET method that we used made it possible to determine the quantity of H. pylori in samples. In all H. pylori infected samples, we found a higher amplification level of ureA gene on area with erosion than those of adjacent area of gastric mucosa. Quantitative results of the H. pylori density (relative rate of ureA/ß-globin) in the stomach and the quantitative results of the UBT were compared and significant correlation was found between these methods. We found many positive results by real-time PCR FRET method, when quantitative results of UBT were unreliable (DOB: 0-4‰).

4. **Investigation of EGFR and PCNA expression:** In H. pylori-positive gastritis with intestinal metaplasia the percentage of EGFR-positive cells was significantly decreased as compared to the normal epithelium and H. pylori-negative gastritis with intestinal metaplasia. A similar, however, not significant difference was detected in chronic gastritis without intestinal metaplasia. The number of PCNA-positive cells was the same in the H. pylori-associated and chronic gastritis group.

5. **Investigation of p53, PCNA and intestinal mucin antigens (SIMA, LIMA) expression:** Intestinal metaplasia (IM) in the adjacent mucosa of chronic gastritis was mostly of incomplete type. Complete type was seen in 23%. The metaplastic areas in 10 gastric epitheli with IM samples showed both SIMA and LIMA. A strong but no significant correlation was found between H. pylori positivity and doubled positive
antigen staining in the gastric epithelium with IM group. A marked significant differences were found between increased expression of p53 and SIMA reactivity in gastric epithelium with IM samples as well as LIMA reactivity. Statistically significant differences were found between IM type I and II in PCNA (p=0.0297) and p53 (p=0.0436) result.

6. **Assessment of intestinal mucin antigens expression and the presence of H.pylori in pediatric biopsy samples:** There was no significant association between the presence of vacA, cagA genotypes and symptoms of abdominal pain, vomiting, loosing weight or hematemesis. IM was sporadically seen in specimens (n=4) by histology, but we demonstrated LIMA and SIMA positivity in 16 pediatric samples. A correlation was found between increased p53 expression and SIMA reactivity in H.pylori infected sample group. Proliferation in chronic gastritis was increased when compared with normal epithelium, but there was no statistical significant difference between chronic gastritis with and without H.pylori measured by PCNA. Apoptotic index increased almost significantly in H.pylori infected chronic gastritis cases compared with normal samples. Expression of p53 was significantly higher in H.pylori-associated gastritis cases compared to chronic gastritis without H.pylori and normal samples groups.

**Conclusions**

1. We established a traditional PCR-based detection method of different genotypes of H.pylori.
2. Detection method of H.pylori cagA, vacA and ureA genotypes was described by real-time PCR assay, using SYBR Green dye and the melting curve analysis.
3. We report the development of a real-time quantitative PCR assay to measure ureA gene copy number to detect H.pylori, based on FRET method.
4. A significant different density of H.pylori was found within the more areas of the same stomach.
5. Comparison of results of DNA-based H.pylori diagnostic techniques with traditional biopsy-based methods suggest that the accuracy of biopsy-based methods for quantitative detection of H.pylori infection seems particularly limited in patients with atrophic gastritis, possibly due to a patchy distribution and reduced number of bacteria. Our observation suggest that the real-time PCR assay is the more sensitive
method than traditional techniques compared with results of our DNA-based diagnostic techniques and biopsy-based methods.

6. A significant difference was found the presence of cagA genotype of H.pylori in gastritis cases and the presence of vacA genotype in gastritis with intestinal metaplasia group, compared with normal gastric mucosa.

7. Proliferating activity was increased in cagA-positive samples compared with cagA-negative specimens.

8. We have not found any significant increase in PCNA labelling index, neither in H.pylori-negative, nor H.pylori-positive gastritis. Our data suggest that increased cell proliferation is not a specific response of the host to cell damage in gastritis, since the number of PCNA-positive cells is equal both in H.pylori-positive and negative gastritis.

9. In our human data the expression of EGFR is decreased in intestinal metaplasia cases, as compared to the normal epithelia. A very similar situation was observed in gastritis specimens.

10. EGFR is a better immunohistochemical marker for the detection of altered gastric epithelial cell function, than investigation of cell proliferation.

11. In adult intestinal metaplasia samples the expression of SIMA and p53 were related to increased severity of epithelial atypia, which suggest that p53 and small intestinal mucin production of epithelium are early events in the multistep process of gastric metaplastic transformation.

12. In pediatric H.pylori infected sample group a correlation was found between increased p53 expression and the presence of cagA genotype.

13. Our results show that in H.pylori-positive gastritis samples the ratio of cells expressing p53 increased considerably and it was followed by a significant increase in SIMA expression.

14. We demonstrated a gradation of aberrant distribution of LIMA and the resurgence of oncofetal antigen (SIMA) in pediatric patients when atypia or metaplasia were absent by microscopic evaluation. Our data contradict Correas mechanism in which intestinal metaplasia is not preceded by chronic gastritis and/or mucosal atrophy, but occurs directly in response to the mucosal damage that induces programmed cell death.
Rudiments of thesis


Ruzsovics A., Molnar B, Tulassay Z.: In situ qualitative-quantitative detection and genotype characterization of Helicobacter pylori by real-time PCR, comparison with non DNA-based methods. Helicobacter (kölész alatt)


Presentations and abstracts connected with thesis

CagA, vacA genotypes of Helicobacter pylori determination from paraffin embedded tissue samples with real-time PCR method.


CagA, vacA genotypes of Helicobacter pylori determination from paraffin embedded tissue samples with real-time PCR method.


Determination of cagA, vacA genotypes of Helicobacter pylori with real-time PCR method.

Ruzsovics A., Molnár B., Tulassay Z.
Determination of H. pylori vacA, cagA and ureaseA genes with real-time PCR analysis and its correlation with immunohistochemistry.

**Ruzsovics A**, Molnár B, Prónaü L, Unger Zs, Tulassay Z:
Z Gastroenterol 2001; 39(5): 417 A149

Different effect of H. pylori on p53 oncoprotein expression and apoptosis of gastric epithelium in presence and absence of intestinal metaplasia.

Unger Zs, Prónaü L, Molnár B, **Ruzsovics A**, Schandl L, Tulassay Z:
Z Gastroenterol 2001; 39(5): 428 A193

Determination of Helicobacter pylori cagA, vacA genotypes with real-time PCR in gastric biopsy specimen. Correlation to proliferation immunohistochemical data.

**Ruzsovics A**, Unger Zs., Ebert M., Molnár B., Prónai L., Malfertheiner P., Tulassay Z:
Digestive Disease Week, Atlanta, 2001, poster of distinction

Comparison of traditional and a quantitative real-time PCR diagnostic techniques for detection of Helicobacter pylori infection in gastric tissues.

**Ruzsovics A**, Molnár B, Prónai L, Tulassay Z:

Quantitative CK20 RT-PCR is in correlation with progression and CA19-9, but not with CEA and CA72-A immunoassays in the follow-up of Dukes D stage colorectal patients.

Molnár B, Floro L, **Ruzsovics A**, Ladányi A, Tulassay Z:
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Comparison of traditional and a quantitative real-time PCR diagnostic techniques for detection of Helicobacter pylori infection in gastric tissues.

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Quantitative CK20 RT-PCR is in correlation with progression and CA19-9, but not with CEA and CA72-A immunoassays in the follow-up of Dukes D stage colorectal patients under chemotherapy.

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Chip technológiák alkalmazása ritka sejtes mintákon.

Molnár B, Galamb O, Sipos F. **Ruzsovics Á**:

Quantitative RT-PCR of peripheral blood cytokeratin 20 (CK20), thymidilate synthase (TS), telomerase related RNA (TELORNA) with tumormarker determinations are useful for the evaluation of responder status to chemotherapy in Dukes stage D CRC patients.
Floro L, Molnár B, Ruzsovics A, Sipos F, Sréter L, Tulassay Z:

Follow-up evaluation of Dukes BC stage patients for the presence of circulating tumor cells by mRNA magnetic cell isolation and serum tumormarkers.
Molnár B, Sipos F, Ruzsovics A, Floro L, Sréter L, Tulassay Z:

Effect of short term omeprasol therapy on the cell proliferation and p53 expression of the gastric epithelia.
Németh A, Ruzsovics A, Molnár B, Sipos F, Prónai L, Tulassay Z:

Assessment of gastric epithelial proliferation, apoptotic activity, p53 and intestinal mucin antigens expression, and cagA, vacA genotypes in H.pylori infected children.
Ruzsovics A, Dezsofi A, Molnár B, Tulassay T, Tulassay Z:

Quantitative RT-PCR of peripheral blood cytokeratin 20 (CK20), thymidilate synthase (TS), telomerase related RNA (TELORNA) with tumormarker determinations are useful for the evaluation of responder status to chemotherapy in Dukes stage D CRC patients.
Floro L, Molnár B, Ruzsovics A, Sipos F, Sréter L, Tulassay Z:

Follow-up evaluation of Dukes BC stage patients for the presence of circulating tumor cells by mRNA magnetic cell isolation and serum tumormarkers.
Molnár B, Sipos F, Ruzsovics A, Floro L, Sréter L, Tulassay Z:

Validation of intestinal mucin antigens in the gastric epithelium with intestinal metaplasia and their relationship with p53 expression and proliferation activity.
Ruzsovics A, Sipos F, Molnar B, Tulassay Z:
Follow-up evaluation of Dukes BC stage patients for the presence of circulating tumor cells by mRNA magnetic cell isolation and serum tumormarkers. 

Quantitative RT-PCR of peripherial blood cytokeratin 20 (CK20), thymidilate synthase (TS), telomerase related RNA (TELORNA) with tumormarker determinations are useful for the evaluation of responder status to chemotherapy in Dukesstage D CRC patients. 

Assessment of gastric epithelial proliferation, apoptotic activity, p53 and intestinal mucin antigens expression, and cagA, vacA genotypes in H.pylori infected children. 

Effect of short term omeprasol therapy on the cell proliferation and p53 expression of the gastric epithelia. 

DNA-based diagnostic techniques to detect H.pylori 

Evaluation of immunhistochemical labelling and detection of intestinal mucin antigens in the gastric epithelium with and without intestinal metaplasia. Correlation to microscopical diagnosis 