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Original Article

Relevance of Ghrelin expression to children with Helicobacter pylori infection

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Abstract: Objective: This study aimed to study the relevance of Ghrelin to children with Helicobacter pylori infection. Methods: 50 children with H.Pylori infection were divided into the anorexia group and the non-anorexia (n=25, each) group according to the clinical manifestation. The changes of the expression of Ghrelin mRNA in gastric mucosa were measured by RT-PCR in the two groups before and after the treatment of H.Pylori. At the same time, the cagA/vacA of H.Pylori isolated from 50 children was further characterized by PCR to determine its genotype. Results: The expression of Ghrelin mRNA in gastric mucosa in anorexia children was significantly increased when compared with that of non-anorexia ($P < 0.01$). The expression of Ghrelin mRNA increased after the treatment of H.Pylori ($P < 0.05$). The symptoms of anorexia children were improved who took food better and gained weight dramatically. The strains of Helicobacter pylori isolated from anorexia and non-anorexia children were type I. The positive rate cagA m1 in the anorexia group was higher than that in the non-anorexia group. More s1/m1 genotypes was found in anorexia group while more s1/m2 genotype was found in non-anorexia group. Conclusions: The expression level of Ghrelin mRNA was decreased in anorexia children with H.Pylori infection and increased after the eradication of H.Pylori. H.Pylori may lead to anorexia through influencing the Ghrelin secretion. Thus, Ghrelin may be the key factor for anorexia children with H.Pylori. Furthermore, different genotypes (vacA s1/m1) may account for the changes of Ghrelin in anorexia children with H.Pylori.

Keywords: Helicobacter pylori (H.pylori), anorexia, virulence genes, ghrelin

Introduction

Clinical symptoms of Helicobacter pylori (H. Pylori) infected children are various. In some cases, there may be no obvious symptoms [1]. While in other cases, patients have poor digestion and are lack of appetite or have symptoms of anorexia [2, 3]. In the worst scenario it can cause chronic gastritis, duodenitis and peptic ulcers, and even lead to parenteral disease. Recently, "anorexia" has been given more attention due to its novel role in causing major complaint or symptom of children, such as chronic malnutrition and delayed growth and development. Researchers in other countries have begun to study the relationship between H.Pylori and anorexia [4]; However, there are no relevant reports in China so far. In this study, we studied a group of non-anorexic children and the H.Pylori infected children who had digestive disorders without serious pathological changes. Clinical studies were carried out to clarify the following issues: The relationship between symptoms of anorexia in H.Pylori infected children with ghrelin secretion (ghrelina gastrointestinal hormone); and the relationship between genotype of the virulence genes of Helicobacter pylori and anorexia.

Patients and methods

Patients inclusion criteria

From June, 2011 to February, 2013, H.pylori infected children taken by the department of Gastroenterology and children from our hospital were collected. The age ranged from 4 to 14-year-old. There were 13 boys and 12 girls in the anorexia group. The mean age was 9.3 ± 0.8 years and a mean duration were 30 ± 11 months. There were 15 boys and 10 girls in the non-anorexia group with an average age of 5 ± 0.8 years and a mean duration of 31 ± 13 months. There was no significant difference in age and gender composition between the two groups ($P > 0.05$). The two groups were comparable.

Table 1. Comparison of height and weight in the two groups of children before and after treatment

Group	n	Height (cm)	Weight (kg)	*t*	*P*
Non-anorexia group	25	114.2 ± 8.5	19.8 ± 1.5	t (Weight) = 2.39	P (Weight) < 0.05
				t (Height) = 2.13	P (Height) < 0.05
Anorexia group	25				
Pre-treatment		107.5 ± 9.1	17.1 ± 1.3	t (Weight) = 2.26	P (Weight) < 0.05
Post-treatment		109.7 ± 7.1	18.9 ± 1.3	t (Height) = 1.81	P (Height) > 0.05

Table 2. Comparison of Ghrelin mRNA expression between H.Pylori infected anorexia patients and non-anorexia patients

Group	n	Ghrelin/β-actin	*t*	*P*
Anorexia group	25	2.86* ± 0.41	3.638	< 0.01
Non-anorexia group	25	3.23 ± 0.39		

*Compared with non-anorexia group, p < 0.01.

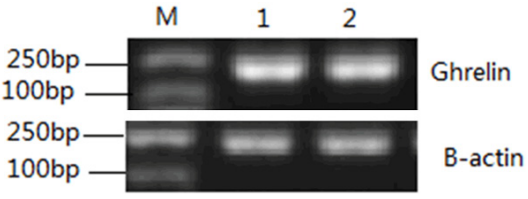

Figure 1. Ghrelin mRNA expression in gastric mucosa. The antral mucosa were prepared and ghrelin mRNA was detected by quantitative RT-PCR.

Patients exclusion criteria

Patients who had the following symptoms or diseases were excluded in this study, including serious infections, heart, lung, kidney or other major organ problems, use of drugs which may affect H.pylori test results (antibiotics, H2 blockers, proton pump inhibitors and bismuth preparations) or corticosteroid. Patients who showed severe gastritis, ulcers or erosion by endoscopy were also excluded.

Groups were divided into two groups according to the diagnostic criteria for anorexia: the experimental group (25 cases of anorexic children with H.Pylori) and the control group (25 cases of non-anorexic children with H.Pylori infection). The diagnostic criteria for anorexia includes long-term loss of appetite, not greedy with food, reduced food intake by 1/3-1/2 compared with premorbid level, exclusion of other diseases, stagnant weight loss and no history of bad eating habits or improper feeding . The duration of no appetite was 2 weeks or more. Efficacy criteria for anorexia were as follows: Cured; disappearance of clinical symptoms, significant increase of food intake (restore the food intake to 2/3 or more of normal levels). Improvement; improvement of clinical symptoms, food intake slightly increased (but below the 2/3 of normal level). Invalid; no significant changes in clinical symptoms and food intake.

Clinical sample preparation

First, Blood testing was performed for the detection of H.pylori antibody in children. Once the screening result of the child was positive, endoscopy was performed and two pieces of antral mucosa were taken. One piece was introduced to bacterial culture and further tested the cagA/vacA gene. The other was used for the detection of Ghrelin mRNA. Regarding to the experimental group, H.Pylori infected children were cured after treatment of a PPI and two types of antibiotics for 2 weeks, after that, there is no further treatment. After one month of withdrawal, two pieces of the relevant children's antral mucosa were taken. One was applied for further culture and the other one was used to detect the Ghrelin mRNA expression. Follow-up of anorexia symptoms, food intake, and physical examination, including height, weight were then performed.

Bacterial culture and identification

Selective Columbia blood agar was used to prepare bacteria culture plate (containing 5 mg/L TMP, 10 mg/L vancomycin, 2500 U/L polymyxin and 2 mg/L amphotericin B). The identification of H.Pylori included colony morphology observation, catalase test, urease test, and smear for the observation of morphology and dyeing properties.

Determination of Ghrelin mRNA expression

Quantitative RT-PCR was used for the detection. (1) Total RNA extraction was performed according to the instructions of Qiagen RNA extraction kit. (2) Reverse transcription: Promega's Reverse Transcription System was applied for the amplification of cDNA in a PCR

Table 3. Comparison of Ghrelin mRNA expression between before and after H.Pylori eradication

Group	n	Ghrelin/β-actin	*t*	*P*
Before H.pylori eradication	25	2.86* ± 0.41	2.25	< 0.05
After H.Pylori eradication	25	3.08* ± 0.39		

*Comparison of Ghrelin mRNA expression between before and after H.Pylori eradication, $p < 0.05$.

Table 4. Expression frequency of cagA m1 in different groups

Group	n	cagA $m1^+$	cagA $m1^-$	χ^2	*P*
Anorexia group	25	86.7%	13.3%	21.6	< 0.001
Non-anorexia group	25	33.3%	66.7%		

cagA $m1^+$: cagA m positive; cagA $m1^-$: cagA m negative.

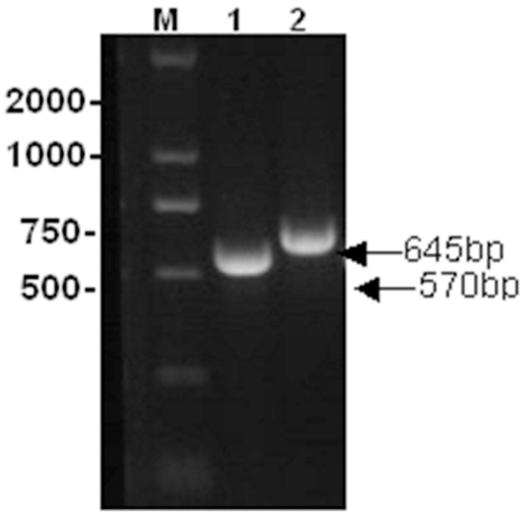

Figure 2. m1/m2 subtype expression of vacA gene. The extracted H.pylori DNA was used as a template for PCR amplification for the detection of H.pylori vacA ml, vacA m2 gene. M: marker; 1: m1; 2: m2.

machine. Sequences of Ghrelin and the housekeeping gene β-actin were searched from the Genebank. All primers were ordered from Shanghai Sangon Biological Engineering Technology And Service Co., Ltd. Primer sequences of Ghrelin were: F1: TGGAGGTCAAGCAGAAGGG R1: GCAGAAGCAAGCGAAAAGC (3) Real-time RT-PCR amplification of the target gene: cDNA was diluted 5-fold in order to prepare standard template based on the initial copy number. 5 μl of each diluted sample template was added to the 30 μl Real-time RT-PCR reaction system. [After 40 cycles of amplification reaction, the system collected the fluorescence intensity of each cycle and analyzed the magnitude of the fluorescence signal generated during the PCR at each time point (DRn) to draw each kinetic curve. Then, determine the cycle number when reached the threshold (Ct value) according to the kinetic curve and calculate the concentration (C value) of the original sample. The ratio of relatively original concentration was obtained after comparing to the Ct value of housekeeping gene β-actin.]

cagA/vacA genotyping

H.pylori in the logarithmic growth phase were collected to extract DNA for PCR quantification. DNA extraction kit (Tiangen. Co., Ltd., Beijing, China) was used to extract genome DNA (stored at -20°C). The extracted H.pylori DNA was used as a template for PCR amplification for the detection of H.pylori cagA, vacA sl, vacA s2, vacA ml, vacA m2 gene. Primers were designed based on previous reports and the detailed information of the primers are listed below: cagA F: 5'-AATACACCAACGCCTCCAAG-3', R: 5'-TTGTTGCCGCTTTTGCTCTC-3' 400 bp, vacA s1/s2 R: 5'-ATGGAAATACAACAAACACAC-3', F: 5'-gcgtctaaataattccaagg-3' s1 259 bp s2 286 bp, vacA m1/m2 F: 5'-gcgtctaaataattccaagg-3', R: 5'-caatctgtccaatcaagcgag-3' m1 570 bp m2 645 bp. The PCR reaction was performed in a total volume of 20 μL including: each primer, 1 μl, 2 × Taq PCR Master Mix10 μl, DNA template, 1 μl, dH_2O 7 μl. PCR amplification of specific steps: 95°C denaturation 5 min, 95°C after denaturation 30 s, 57°C annealing 30 s, 72°C extension of 2 min for 30 cycles, 72°C for 10 min. The PCR product was then run on 2% Agarose gel at 100 V for 45 minutes.

Statistical analysis

All data were expressed as $\bar{X} \pm s$. T-test was used for the comparison of two sets of data. Chi-square (χ^2) test was used to compare the ratio. $P < 0.05$ was considered to be significantly different.

Results

The results of comparison of appetite improvement, height and weight after H.Pylori eradication were as follows: 62% of the patients have improved their appetite remarkably, 31% of them have showed effective improvement, 7% has showed no improvement. Thus, the total effective rate was 93%. There were significant differences between the non-anorexia group

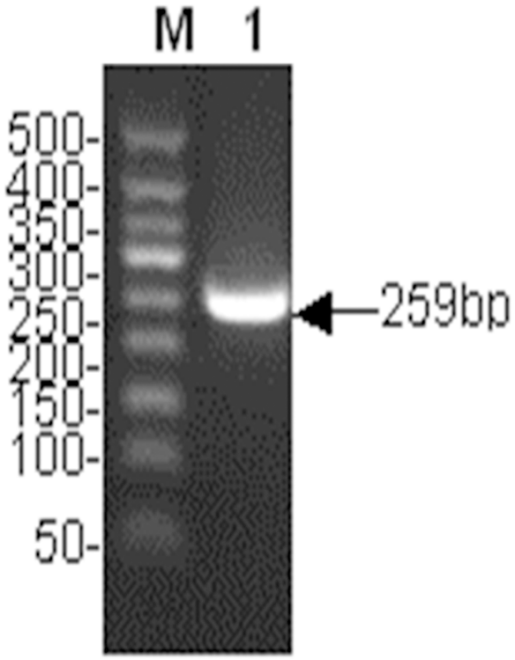

Figure 3. s1 subtype expression of vacA gene. The extracted H.pylori DNA was used as a template for PCR amplification for the detection of H.pylori vacA sl. M: marker; 1: s1.

and the anorexia group in weight and height ($P < 0.05$). In the anorexia group, the body weight of children significantly increased after H.Pylori eradication ($t=2.26$, statistically significant differences between before and after treatment, $P < 0.05$). However, height growth was insignificant ($t=1.81$, $P > 0.05$, **Table 1**).

Comparison of Ghrelin mRNA expression between H.Pylori infected anorexia patients and non-anorexia patients

In the anorexia group, the expression of gastric Ghrelin mRNA was significantly lower than that of non-anorexia group ($P < 0.01$, **Table 2**). Ghrelin mRNA was detected by RT-PCR as shown in **Figure 1**.

Comparison of Ghrelin mRNA expression between before and after H.Pylori eradication. gastric Ghrelin mRNA levels significantly increased after H.Pylori eradication ($P < 0.05$). The difference was statistically significant as shown in **Table 3**.

Expression rate of H.Pylori genotype in anorexia and non-anorexia groups: cagA and vacA gene were both expressed in patients of anorexia group and non-anorexia group. The H.Pylori strain was type I. s1-positive rate was 100% in the anorexia group and non-anorexia group while all the cases showed no expression of s2. The positive expression rate of cagA m1 in the anorexia group was significantly higher than that in the non-anorexia group ($P < 0.001$). In the anorexia group, the genotype was mainly s1/m1 which is different from the s1/m2 genotype in the non-anorexia group (**Table 4**). PCR amplification results of the Helicobacter pylori gene were shown in **Figures 2** and **3**.

Discussion

It is well known that H.Pylori infection in adults is the main pathogenic factor in developing chronic gastritis, duodenal ulcer, gastric cancer, gastric mucosa-associated lymphoma and other relative diseases. In the meantime, H. Pylori infection is closely related to the occurrence and development of chronic gastritis, duodenitis and peptic ulcer in children [1, 2]. Epidemiological studies indicated that there might be no obvious symptoms in the early stage of H.Pylori infection in children. As the disease manifests, it shows poor digestion and lack of appetite or symptoms of anorexia and lead to gastrointestinal pathological changes. Because of that, “anorexia” is recognized as major complaints, and draws more attention due to the early clinical manifestations of H.Pylori infection. It is necessary to clarify the mechanism of anorexia induced by H.Pylori infection pathogenesis so that timely and effective treatment for anorexia can be implemented to improve the nutrition and physical development of children. Moreover, aggragation of H.Pylori infection or related diseases can be prevented [3, 4].

Anorexia in H.Pylori-infected children can occur before pathological changes of gastrointestinal tract which indicates that there might be functional pathogenesis in children with anorexia. Gastrointestinal hormone Ghrelin is a recently reported gut-brain peptide that regulates food intake of human [5, 6]. It is mainly synthesized and secreted by gastric endocrine cells. Ghrelin is composed of 28 amino acids and has been reported to not only stimulate the hypothalamus appetite center through the neuropeptide YNPY/AGRP, but also directly regulate gastrointestinal function and appetite [7, 8]. Ghrelin also enhances the motility of gastrointestinal

tract and accelerates gastric emptying which improves the appetite [9-11]. In our study, we chose patients without serious pathological changes and serious injuries, such as erosion, ulcers and other gastrointestinal injury. We found Ghrelin mRNA expression was at low levels in children with anorexia. However, after anti-H.Pylori treatment, Ghrelin, weight and appetite were all significantly increased ($P < 0.05$). Height growth change was not obvious ($P > 0.05$) which might be related to the shorter observation time, which implied that H.Pylori infection affected normal secretion of Ghrelin, in turn, caused anorexia in children. Meta-analysis on Helicobacter pylori infection performed by Nweneka [12] and most studies have confirmed that H.Pylori can cause the secretion of Ghrelin and H.pylori-positive subjects had significantly lower ghrelin blood levels than that of non-infected individuals. Moreover, Ghrelin secretion is related to the duration of infection, the degree of mucosal inflammation and destruction of Ghrelin-secreting cells.

What is the effect of the Ghrelin secretion by H.Pylori? Studies have shown that H.Pylori infection causes tissue inflammation and release of many inflammatory cytokines, such as IL-1, IL-6, IL-8. Furthermore, it also induces cell swelling, degeneration, necrosis and destruction of Ghrelin-secreting cells which leads to the reduction of Ghrelin secretion [13, 14]. Abiko et *al* [15] further reported the relationship between H.Pylori infection, Ghrelin secretion and appetite by using IL-1 gene knockout mice. In his study, by comparing the Ghrelin level, appetite and body weight in the H.Pylori infected IL-1 gene knockout mice with those in wild-type mice, he found that there was no obvious change in knockout mice. However, in wild-type mice, the Ghrelin level, appetite and body weight decreased significantly indicating that H.Pylori infection can affect the inflammatory response of Ghrelin secretion. Clearly, the obvious change in the inflammatory response is associated with the virulence of H.Pylori strains. Strong virulence of H.Pylori induced strong infiltration of inflammatory cells and strong inflammatory response.

Fifty H.Pylori-positive patients in our study were classified to H.Pylori infection of type I (cagA and vacA positive). In addition, genetype of most patients in the anorexia group was s1/m1 which had a stronger virulence than that in non-anorexia group whose genotype was s1/m2. Our results were similar with other studies [16, 17] and we confirmed that a stronger virulence of H.Pylori leads to a lower level of Ghrelin secretion.

In short, our results suggested that the mechanism of anorexia in H.Pylori infected children was that a strong virulence apparatus within H.Pylori was more prone to cause severe local inflammation in the gastric mucosa and Ghrelin-secreting cells injury, reduce the secretion of Ghrelin and lead to poor appetite or anorexia.

Disclosure of conflict of interest

None.

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