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Association between *Helicobacter pylori* Infection and Telomere Length: Effect of Eradication Therapy

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Authors' contributions

This work was carried out in collaboration between all authors. Authors AAA and RRAEG are equally contributed in planning and designing the study, collecting the samples, performing the practical laboratory activities, analyzing the results, drafting and revising the manuscript. Author MZO participated in planning and designing the study, clinical evaluation of patients and controls and participated in the interpretation of the results. The three authors read and approved the final manuscript.

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ABSTRACT

Aims: This study aimed to assess the relative telomere lengths (TLs) in gastric mucosa of patients infected with *Helicobacter pylori (H. pylori)* compared to *H. pylori*-negative controls and determine the effect of *H. pylori* eradication therapy on TL.
Place and Duration of Study: This is a seven months case- control study conducted in Hepatology, Gastroenterology and Infectious Diseases and Medical Microbiology & Immunology Departments, Benha University, Egypt.
Methodology: Relative TLs in gastric mucosa were analyzed by Real Time-Polymerase Chain Reaction (RT-PCR) in 15 *H. pylori* -positive patients (Group I: 10 patients with gastric ulcer, Group II: 5 patient without gastric ulcer) and 10 *H. pylori* -negative controls (Group III). Relative TLs were re-evaluated in *H. pylori*-positive patients 4 weeks after *H. pylori* eradication therapy.
Results: Highly significant shortening (P<0.001) was observed in TLs in gastric mucosa of *H. pylori*

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– positive patients compared to *H. pylori* – negative controls. Highly significant elongation (*P*<0.001) was observed after *H. pylori* eradication therapy. This elongation was significant in both group I and II (*P* <0.001, 0.01).
Conclusion: *H. pylori* -positive patients had significantly shorter TLs than *H. pylori* negative controls. TLs were increased after *H. pylori* eradication therapy in all patients either with or without gastric ulceration and could be considered as one of preventable methods for gastric cancer.

Keywords: Helicobacter pylori; telomere length; real time PCR; gastric ulcer.

1. INTRODUCTION

Telomeres are specialized structures composed of nucleotides that are located at the ends of eukaryotic chromosomes. They cap the termination of double strands of DNA and thus preserve the integrity and stability of the genome during duplication [1]. Short telomeres are associated with cellular senescence and decreased tissue renewal capacity [2]. Longer telomere length (TL) appears to prevent genomic instability and development of cancer in human aged cells by limiting the number of cell divisions. However, shortened telomeres impair immune function that might also increase cancer susceptibility [3].

Meta-analyses suggest 1.4 to 3.0 fold increased risk of cancer for those with the shortest versus longest telomeres [4,5].

Inflammation, oxidative stress and increased cell replication are major environmental factors associated with accelerated shortening of telomeres [6].

Several studies have reported that certain forms of gastrointestinal cancers are associated with telomere shortening. Relative telomeric repeat content was substantially reduced in inflamed liver tissues as well as in hepatocellular carcinoma [7]. Telomere shortening was also noted in colon biopsies from ulcerative colitis patients with low grade dysplasia [8]. Persistent microbes can contribute to the progressive shortening of telomeres in blood leukocyte genomic DNA [9, 10].

Helicobacter pylori (H. pylori) infection is a known risk factor for chronic gastritis, gastric ulcers and gastric cancer. Chronic inflammation mediated by such infection is closely linked with TL shortening [11]. The risk of gastric cancer was increased by 2-fold in *H. pylori*-infected patients, who had shortened telomere [12].

Shorter TL was identified in *H. pylori*–positive gastric epithelial tissue [13] and tumor tissues [14] compared with normal gastric tissues.

This study aimed to assess the relative TLs in gastric mucosa of patients infected with *H. pylori* compared to *H. pylori*-negative controls and determine the effect of *H. pylori* eradication therapy on TL.

2. MATERIALS AND METHODS

2.1 Patients and Controls

This study was carried out in the Hepatology, Gastroenterology and Infectious Diseases and Medical Microbiology and Immunology Departments, Faculty of Medicine, Benha University from February 2015 to September 2015. The study was conducted on 25 dyspeptic patients (defined symptomatically as abdominal discomfort related to meals) attending the out patients clinics of Hepatology, Gastroenterology and Infectious Diseases. According to the results of upper GIT endoscopy, biopsy urease test and microbiological culture, selected patients were divided into 3 groups. Group I: (10) *H. pylori* – positive patients with ulcer or erosions .Group II: (5) *H. pylori* -positive patients without ulcer or erosions. Group III: (10) *H. pylori* –negative patients with functional dyspepsia (according to Rome II criteria) [15] and they were considered as a control group.

All patients with hepatic, pulmonary, renal and cardiac diseases or with contraindication to endoscopy were excluded from the study. All studied patients did not receive non-steroidal anti-inflammatory drugs (NSAIDs), proton pump inhibitors (PPI) or antibiotics within the previous two months. All studied patients were subjected to: Full history taking, complete clinical examination, complete blood picture, kidney function tests (urea and creatinine), liver function tests (SGOT, SGPT, serum bilirubin, serum albumin, and prothrombin time).

The study was approved by the local ethics committee of Benha University Hospitals and written consent was taken from each participant.

2.2 Sampling

Upper GIT endoscopy was done by the same operator: to evaluate for the presence of gastritis, erosions or ulcers. Four quadrant biopsies were taken from antral mucosa within 5 cm of the pyloric opening for detection of *H. pylori* infection using urease test and microbiological culture for *H. pylori* and TL analysis. In positive *H. pylori* cases upper GIT endoscopy was repeated one month after *H. pylori* eradication therapy (a full dose PPI, Clarithromycin 500 mg and Metronidazol 400 mg, all twice daily for 2 weeks). Gastric fragments were kept in thioglycolate broth (Difco Laboratories, Detroit, Mich.) at 4℃. Samples for TL analysis were sent to the laboratory in tubes with 0.9% NaCl. Then they were centrifuged at3000*g* for 5 minutes, the supernatant was removed, and the tissue samples were stored at -80℃ until TL was analyzed.

2.3 Identification of *H. pylori*

2.3.1 Urease test

Biopsies were placed in tubes containing christensen's 2% urea agar and examined within 24 h of incubation at 37℃ for urea hydrolysis.

2.3.2 Microbiological culture

Fragments in thioglycolate broth (Difco Laboratories, Detroit, Mich.) were ground in a tissue homogenizer. Biopsies were rubbed onto Dent's agar plates using Columbia agar base supplemented with 7% human blood containing vancomycin, trimethoprim, cefsulodin and amphotercin B (Oxoid, Basingstoke, United Kingdom). Plates were incubated for 4-7 days in microaerophilic environment at 37℃. Bacteriological identification of *H. pylori* was done. Patients considered positive for *H. pylori* if direct urease and culture were positive or if culture alone was positive and negative if both tests were negative [16].

2.4 Genomic DNA Extraction

DNA was extracted using QIAamp DNA mini kit (Qiagen, Germany) according to the manufacturer instructions. The extracted DNA concentration was confirmed through measurement by NanoDrop 2000 c Spectrophotometer. Readings were taken at wave lengths of 260 and 280 nm. Concentration of DNA samples were measured = 30 ng /µL at wave lengths 260 and 280 nm [17].

2.5 Quantitative Real Time PCR

As described by Cawthon [18].

2.5.1 Principle ot the test

The relative TLs for each DNA sample derived from gastric tissue was measured as the ratio of telomere hexamer repeat (THR) copy number to autosomal single copy gene (SCG) copy number. The ratio (THR/SCG) is proportional to the TL if the number of copies of SCG /cell is the same in all studied samples. The relative ratio of SCG copies to the housekeeping gene human β-globin (HBG) copies in expirermental DNA versus reference DNA was determined. The ratio $\Delta C_t^{SCG} / \Delta C_t^{\beta\text{-globin}}$ for all samples should be 1±0.05 indicatig that equal copy numbers of SCG per cell were amplified in all DNA samples.

The autosomal single copy 36B4 gene, encoding acidic ribosomal phosphoprotein, is located on chromosome 12. This gene was chosen because it has been validated for gene dosage studies [19].

2.5.2 Preparation of reference DNA sample

The reference DNA sample was prepared from pooled DNA samples. Pooled DNA was created using DNA from the 10 control subjects distributed into aliquots. Serial dilutions of a reference DNA sample were used to generate a fresh standard curve in separate THR and SCG PCR run. For each standard curve, one reference DNA sample was serially diluted in TRIS EDTA buffer (TE) by 1:2 folds per dilution to produce six concentrations of DNA ranging from 0.47 to15 ng /µL. Five µL of each concentration was distributed to the standard curve tubes of each amplification run.

2.5.3 Amplification protocol

Amplification was performed in 36-well rotor (Rotor-Gene Q 5plex. Qiagen, Germany) using Rotor-Gene_2_3_1_software. The THR and SCG PCRs were prepared identically with the exception of the oligonucleotide primers.

In each reaction, 12.5 µl of the Super Real Pre Mix Plus (SYBR Green) (Tiangene, Biotech) master mix were added to each amplification tube, 0.75 µl (0.3 µM) of forward primer, 0.75 µl (0.3 µM) of reverse primer and 5 µl of DNA template and completed with RNAse- free water to 25 µL.

Table 1. Primer sequences in amplification reaction [18]

Gene	Primers	
	Forward	**Reverse**
THR*	5'-GGTTTTTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGT-3'	5'-TCCCGACTATCCCTATCCCTATCCCTATCCCTATCCCTA-3'
36B4**	5'-CAGCAAGTGGGAAGGTGTAATCC-3'	5'-CCCATTCTATCATCAACGGGTACAA-3'
HBG***	5'-GCTTCTGACACAACTGTGTTCACTAGC-3'	5'-CACCAACTTCATCCACGTTCACC-3'

**Telomere hexamer repeats, **36B4 gene, encoding acidic ribosomal phosphoprotein, ***human β-globin*

In Rotor-Gene Q 5plex (Qiagen, Germany), the thermal cycling condition for THR PCR was 95℃, 15 min for initial denaturation followed by 30 repeated cycles of 95℃, 30 sec; 54℃ ,1 min.; 72℃, 30 sec for denaturation, annealing and extension respectively. The cycling condition for both the autosomal SCG 36B4 and housekeeping HBG was 95℃, 15 min for initial denaturation followed by 30 repeated cycles of 95℃, 30 sec; 58℃, 1 min.; 72℃, 30 sec for denaturation, annealing and extension respectively. The primers used in amplifications (Fermentas, Germany) were described in Table1 (above).

At the end of each reaction, a melting curve was used for both THR and SCG PCRs. All samples were run in duplicate and the mean of two measurements was used in the statistical analyses.

2.5.4 Standard curve analysis

A plot of C_T (the fractional cycle number at which the well's accumulating fluorescence crosses a set threshold that is several standard deviations above baseline fluorescence), versus log (amount of input target DNA) is linear, allowing simple relative quantitation of unknowns by comparison to a standard curve in the same PCR run. The quantity of telomere repeats or SCG in each experimental sample was measured as the level of dilution of the calibrator DNA sample that would make the experimental and calibrator samples equivalent with regard to the C_T during the exponential phase of PCR amplification. The average TL for each sample was measured by comparing the corresponding dilution of the sample's THR PCR product to the dilution of the same sample's SCG PCR product to compute the T/S ratio. Repeated measures of the T/S ratio in the same DNA sample gave the lowest variability when the sample well for THR PCR on the first PCR run matched its well position for SCG PCR on the second run.

2.6 Statistical Analysis

The collected data were summarized in terms of mean ± Standard Deviation (SD) and range for quantitative data and frequency and percentage for categorical data. Comparisons between the studied groups were carried out using the Fisher's Exact Test (FET), the Student's t-test (t) as appropriate. Comparisons between *H. pylori* - positive patients before and after treatment were carried out using the paired t-test (t). A *P*-value <0.05 was considered statistically significant. The statistical analysis was conducted using STATA version 11 (STATA corporation, College Station, Texas).

3. RESULTS

This study was conducted on 15 *H. pylori*-positive patients (11 males and 4 females) with mean age 39.4±7.85 years and 10 *H. pylori*-negative patients as a control group (8 males and 2 females) with mean age 38.67±7.83 years. Patients and controls were classified into four age groups. Insignificant statistical difference (*P*=1.00) was detected between patients and controls as regard to age and sex, Table 2.

Highly significant shortening (*P*<0.001) was observed in TLs in *H. pylori* - positive patients compared to *H. pylori* - negative controls (0.41±0.13 vs1.1±0.36). Also significant shortening (*P*= 0.03, 0.01, 0.01) was found in TL in *H. pylori* -positive patients than controls in different age groups: ≤30 years (0.42±0.09 vs1.1±0.42), 31-40 years (0.4±0.12 vs1.03±0.42), 41-50 years (0.4±0.19 vs1.15±0.46) and >50 years (0.4±0.0 vs1.1±0.0), Table 3.

Interestingly, after *H. pylori* eradication therapy, highly significant elongation (*P*<0.001) in TLs (0.89±0.21 versus 0.41±0.13) was observed in total *H. pylori* –positive patients. This statistical significant elongation was observed (*P*=0.01, 0.03, 0.03) in all different age groups: ≤30 years (1±0.11vs0.42±0.09), 31-40 years (0.96±0.28 vs 0.4±0.12), 41-50 years (0.76±0.11vs 0.4±0.19) and >50 years (0.7±0.0 vs 0.4±0.0), Table 4.

On the other hand, insignificant statistical difference (*P*=0.07) was detected in TL between total *H. pylori* -positive patients after treatment and controls (0.89±0.21 versus1.1±0.36). The same finding was observed (*P*=0.65, 0.77, 0.11) in all age groups: ≤30 years (1±0.11vs 1.1±0.42), 31-40 years (0.96±0.28 vs1.03±0.42), 41-50 years (0.76±0.11vs1.15±0.46) and >50 years (0.7±0.0 vs 1.1±0.0), Table 5.

TLs were notably increased in both groups I and II of *H. pylori*- positive patients after the eradication therapy. This increase was significantly higher in group I than group II (*P* <0.001,0.01) (0.89±0.17 vs 0.4±0.07), (0.75±0.12 vs 0.45±0.14) respectively, Table 6.

4. DISCUSSION

Telomeres are special chromatin structures that protect the ends of chromosomes from degrading and restructuring activities [20]. They consist of repetitive nucleotide sequences and an associated terminal protein complex that is vital for chromosomal stability, replication and prevent loss of chromosomal integrity [21]. Telomere shortening results in the deterioration of the protective functions, fusion in chromosomes, breaking and bridging, and gene amplifications. All of which lead to genomic instability, the most significant feature of solid tumors [22].

Decline of TL occurs with different dynamics in different human individuals as well as different human organs [23]. Telomere shortening in genomic DNA appears to reflect lifetime cumulative oxidative stress from environmental exposures, such as smoking, poor nutrition, and chronic inflammation [24-26].

Table 2. Comparison between cases and controls regarding sex and age

Variable	Cases (No.=15)	Control (no.=10)	Test	P
Sex			FET	1.00
Females (%)	4 (26.67)	2 (20.00)		
Males (%)	11 (73.33)	8 (80.00)		
Age (years)				
Mean±SD; (range)	39.4±7.85; (25-52)	38.67±7.83; (25-52)	t= 0.23	0.82
≤30	4 (26.67)	2 (20.00)	FET	1.00
31-	5 (33.33)	3 (30.00)		
41-	5 (33.33)	4 (40.00)		
>50	1 (6.67)	1 (10.00)		

Table 3. Relative TLs among different age groups in *H. pylori*-positive patients before treatment compared to controls

Study groups				Relative TL*				
				Mean±SD		Range		
H. pylori **-positive patients before treatment (No.=15)				0.41±0.13		0.2-0.6		
H. pylori- negative control group (No.=10)				1.1±0.36		0.5-1.6		
T				6.81				
P				<0.001				
Age (years)	**Relative TL in *H. pylori*-- positive patients before treatment (No.=15)**			**Relative TL in control group (No.=10)**			**t**	**P**
	Mean±SD	**Range**	**No.**	**Mean ±SD**	**Range**	**No.**		
≤30	0.42±0.09	0.3-0.5	4	1.1±0.42	0.8-1.4	2	3.42	0.03
31-	0.4±0.12	0.3-0.6	5	1.03±0.42	0.7-1.5	3	3.33	0.01
41-	0.4±0.19	0.2-0.6	5	1.15±0.46	0.5-1.6	4	3.33	0.01
>50	0.4±0.0	0.4	1	1.1±0.0	1.1	1	-	-

**Telomere length, **Helicobacter pylori*

Table 4. Relative TLs among different age groups in *H. pylori* - positive patients before and after eradication therapy

Study groups (No.=15)	Relative TL*	
	Mean±SD	Range
*H. pylori***- positive patients before eradication therapy	0.41±0.13	0.2-0.6
H. pylori -positive patients after eradication therapy	0.89±0.21	0.6-1.3
T	6.74	
P	<0.001	

Age (years)	Relative TLs in *H. pylori*- positive patients before eradication therapy (No.=15)		Relative TLs in *H. pylori*- positive patients after eradication therapy (No.=15)		T	*P*
	Mean±SD	Range	Mean±SD	Range		
≤30 (No.=4)	0.42±0.09	0.3-0.5	1±0.11	0.9-1.1	5.58	0.01
31- (No.=5)	0.4±0.12	0.3-0.6	0.96±0.28	0.7-1.3	3.43	0.03
41- (No.=5)	0.4±0.19	0.2-0.6	0.76±0.11	0.6-0.9	3.34	0.03
>50 (No.=1)	0.4±0.0	0.4	0.7±0.0	0.7-0.7	-	-

**Telomere length, **Helicobacter pylori*

Table 5. Relative TLs among different age groups in *H. pylori*- positive patients after eradication therapy compared to controls

Study groups	Relative TLs*	
	Mean±SD	Range
H. pylori **-positive patients after eradication therapy (No.=15)	0.89±0.21	0.6-1.3
Control group (No.=10)	1.1±0.36	0.5-1.6
T	1.87	
P	0.07	

Age (years)	Relative TLs in *H. pylori* positive patients after eradication therapy (No.=15)			Relative TLs in control group (No.=10)			t	P
	Mean±SD	Range	No.	Mean±SD	Range	No.		
≤30	1±0.11	0.9-1.1	4	1.1±0.42	0.8-1.4	2	0.49	0.65
31-	0.96±0.28	0.7-1.3	5	1.03±0.42	0.7-1.5	3	0.30	0.77
41-	0.76±0.11	0.6-0.9	5	1.15±0.46	0.5-1.6	4	1.83	0.11
>50	0.7±0.0	0.7-0.7	1	1.1±0.0	1.1	1	-	-

**Telomere length, **Helicobacter pylori*

Table 6. Relative TL in group I and II *H. pylori* -positive patients before and after eradication therapy

Relative TLs* in *H. pylori***- positive patients group I before eradication therapy (No.=10)		Relative TLs in *H. pylori*- positive patients group I after eradication therapy (No.=10)		T	*P*
Mean±SD	Range	Mean±SD	Range		
0.4±0.07	0.3-0.5	0.89±0.17	0.7-1.1	8.43	<0.001
Relative TLs in *H. pylori*- positive patients group II before eradication therapy (No.=5)		**Relative TLs in *H. pylori*- positive patients Group II after eradication therapy (No.=5)**		**T**	***P***
Mean±SD	**Range**	**Mean±SD**	**Range**		
0.45±0.14	0.3-0.7	0.75±0.12	0.6-0.9	3.64	0.01

**Telomere length, **Helicobacter pylori, Group I: H. pylori –positive patients with ulcer Group II: H. pylori –positive patients without ulcer*

Pre-neoplastic changes that might progress into gastric cancer are found in around 50% of people infected with *H. pylori* [27] and TL has been found to be shorter in some tumors as colon cancer and gastric cancer compared to normal tissue [5,28].

This study aimed to assess the relative TLs in gastric mucosa of patients infected with *H. pylori* compared to *H. pylori*-negative controls and to evaluate the effect of *H. pylori* eradication therapy on TL.

Highly significant shortening (P<0.001) in TLs was detected in *H. pylori* - positive patients than controls in addition to significant shortening (P= 0.03, 0.01, 0.01) in TL in *H. pylori* -positive patients than controls in different age groups (P= 0.03, 0.01, 0.01). This finding is in accordance with Aida et al. [13] who demonstrated that *H. pylori*–positive gastric mucosa has been shown to have shorter TL than *H. pylori*–negative mucosa.

Similarly, Kuniyasu et al. [29] found that TL was significantly shorter in the group infected by *H. pylori* than in the uninfected group.

As the extent of telomere shortening may vary considerably among individuals within age groups, suggesting that environmental and lifestyle factors could play critical roles in the rate of telomere attrition [12]. The effect of age in our study is hidden by the effect of *H. pylori* infection which contributes to various degrees of telomeres shortening according to severity of the condition.

Previous studies reported that carriage of gastric *H. pylori*, like other persistent microbes can contribute to the progressive shortening of telomeres in blood leukocyte genomic DNA [9,30,12]. *H. pylori* infection may also facilitate telomere shortening process by increasing cumulative oxidative stress [25] that has been proposed as a potential mechanism for *H. pylori* infection-related gastric cancer [31].

More interestingly, after *H. pylori* eradication therapy, highly significant elongation (P<0.001) in TLs was observed in total *H. pylori* –positive patients, also significant elongation was observed (P=0.01, 0.03, 0.03) in different age groups. This finding provides a new platform for the effectiveness of *H. pylori* eradication therapy in increasing the TL. Similar results were previously reported as TLs were significantly increased after eradication therapy of *H. pylori* and this increase is related to a decrease in mucosal oxidative stress after *H. pylori* eradication [32].

Elongation of TL was more notable in *H. pylori*-positive patients with ulcer (group I) than those without ulcer (group II) (p <0.001, 0.01) respectively. Chung et al. [21] elucidated that peptic ulcer and intestinal metaplasia are closely related to *H. pylori* infection. Furthermore, chronic gastritis due to *H. pylori* infection may progress to intestinal metaplasia and even gastric cancer.

Tuo et al. [33] suggested that telomere shortening may lead to a relative imbalance of the protective secretory capacity and the aggressive secretory products in the upper gastrointestinal tract. This may be one of the factors predisposing to duodenal ulcer development in advanced age.

Nagata et al. [34] showed that *H. pylori* itself could produce superoxide. This production is performed by electrons leaking during electron transport from mitochondria. In Dülger et al.'s study [35] total oxidant state, oxidative stress index, and peripheral lymphocyte DNA damage in *H. pylori* -positive patients were found to be higher than in *H. pylori* -negative patients and they were decreased after *H. pylori* eradication.

Telomeres comprise simple, repetitive and G-rich hexameric sequences (TTAGGG)[21].This high guanine content in specific telomeric sequences, makes them remarkably sensitive to damage by oxidative stress and telomeric DNA is deficient in the repair of single-strand breaks induced by oxidative DNA damage [6,36].

Epidemiologic evidence indicates that *H. pylori* infection increases the risk of gastric carcinoma. *H. pylori* infection leads to chronic atrophic gastritis, which frequently advances to intestinal metaplasia, occasionally to dysplasia, and carcinoma. *H. pylori* infection increases the rate of proliferation of the gastric epithelial cells and decreases the gastric secretion of ascorbic acid, processes that may modulate the process of carcinogenesis [37].

5. CONCLUSION

H. pylori -positive patients have significantly shorter TLs in their gastric mucosa compared to controls. *H. pylori* eradication therapy increases TL in all patients either with or without gastric ulceration and could be considered as one of preventable methods for gastric cancer. Further large scaled studies are recommended to confirm the role of telomere shortening in *H. pylori* associated carcinogenesis.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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