Impact of *Helicobacter pylori* Eradication on Absolute Telomere Length in Gastric Mucosa

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Background and study aim: This study proposed to assess the relation between absolute telomere lengths (TLs) in gastric mucosa and *Helicobacter pylori* (*H. pylori*) infection and study the impact of (*H. pylori*) eradication therapy on TLs.

Patients and Methods: This study was conducted on (42) subjects divided into two groups, group I: included (17) *H. pylori* negative cases served as control group, group II: included (25) *H. pylori* positive patients. Absolute TLs was measured in base pairs (bp) in gastric mucosa and analyzed by real time polymerase chain reaction (RT-PCR) in all groups and re-evaluated in *H. pylori* positive patients 4 weeks after eradication therapy.

Results: Prior to eradication therapy, there was highly significant shortening (P<0.001) in TLs (bp) in gastric mucosa of *H. pylori* positive patients compared to *H. pylori* negative controls. While there was highly significant elongation was observed after *H. pylori* eradication therapy in H. pylori positive patients (P < 0.001). There was significant negative correlation between TLs and ages of patients (P<0.001) while there was no significant relation between TLs and sex (P= 0.5) before and after eradication therapy.

Conclusion: *H. pylori* positive patients had significantly shorter TLs than *H. pylori* negative controls. TLs were increased after *H. pylori* eradication therapy in H. pylori positive cases. This finding may indicate the importance of *H. pylori* eradication to avoid the development of gastric cancer by its effect on TLs.

INTRODUCTION

Helicobacter pylori (*H. pylori*) is a gram- negative, non-invasive, helix-shaped, microaerophilic agent. It is the most common chronic bacterial infection in humans. *H. pylori* infection is strongly related with socioeconomic status and its prevalence is over 80% in developing countries and 20-50% in developed countries [1]. *H. pylori* is the etiological agent of chronic gastritis, peptic ulcer, and has been documented to be linked with the development of gastric adeno-carcinoma and lymphoma [2]. Histological pre-neoplastic changes that might progress into gastric cancer are found in around 50% of people infected with *H. pylori*. Despite this fact, less than 2% of the patients develop gastric cancer [3]. Although many factors may be related to *H. pylori* associated gastric carcinogenesis, the underlying molecular mechanisms are still unknown. The complications of chronic inflammation associated with *H. pylori* infection are believed to be related to the genetic features of the host, virulence factors of the bacteria strain, and environmental factors [4]. Telomerase, located at the distal end of human chromosomes, comprises simple, repetitive and G-rich hexameric sequences (TTAGGG), and is vital for chromosomal stability and replication [5]. Short telomeres are associated with cellular senescence and decreased tissue renewal capacity [6]. 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 [7]. Telomere length has been analyzed in many human cancers, and it has been found to be shorter in some tumors

(e.g. colon cancer, gastric cancer and glioblastoma) and longer in others (e.g. chordoma) compared to normal tissue **[8,9]**. In this study we aimed to assess the absolute (TLs) in gastric mucosa of H. pylori positive patients compared to H. pylori negative controls and to determine any changes in (TLs) by *H. pylori* eradication therapy.

PATIENTS AND METHODS

Patients:

This cohort prospective study was carried out in the Hepatology, Gastroenterology and Infectious Diseases and Medical Microbiology and Immunology Departments, Faculty of Medicine, Benha University from January 2016 to July 2016. The study was approved by the local ethics committee of Benha University Hospitals and written consent was taken from each participant. Forty two adult patients were enrolled in this study were complaining of dyspeptic symptoms (defined as abdominal pain related to the meals). According to the results of upper GIT endoscopy, biopsy urease test and microbiological culture, selected patients were divided into 2 groups. Group I: (17) *H. pylori* negative patients with functional dyspepsia (according to Rome II criteria) **[10]** and they were considered as a control group. Group II: (25) *H. pylori* positive patients. Patients with chronic disease as liver cirrhosis, chronic renal failure, diabetes, hypertension, obstructive airway diseases, patients with history of non-steroidal anti-inflammatory drugs (NSAIDs), proton pump inhibitors (PPI) within previous month, heavy smokers and patients with cancers were excluded from the study.

All patients were subjected to full history taking, thorough clinical examination and routine laboratory investigation.

Upper GIT endoscopy: was done for all patients and controls by the same endoscopist after fasting at least 8 hours using video endoscope (OLYMPUS GIF TYPE Q180 2001629, Japan). 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 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 Metronidazole 400 mg, all twice daily for 2 weeks) and biopsy samples were again taken to determine the changes in telomere length after eradication therapy, gastric fragments were kept in thioglycolate broth (Difco Laboratories, Detroit, Mich.) at 4°C. Samples for TL analysis were sent to the laboratory in tubes with 0.9% NaCl. Then they were centrifuged at 3000g for 5 minutes, the supernatant was removed, and the tissue samples were stored at -80°C until TL was analyzed using (RT.PCR).

Identification of *H. pylori*

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.

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°C. 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 considered negative if both tests were negative **[11]**.

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 2000c 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 **[12]**.

Quantitative real time PCR: as described by Cawthon **[13]**.

Telomere length assay

Telomere and human β- Globin (HBG) gene were analyzed on DNA samples in 36-well rotor (Rotor-Gene Q 5plex. Qiagen, Germany) using Rotor-Gene_2_3_1_software.

HBG was used as a single copy gene needed for normalization. Necessary standards for absolute quantitation of β-globin expression were prepared using QuantiTect SYBR Green PCR Kit (Roche, Germany).

The telomere length assay was performed according to manufacturer's instructions with a Telo TAGGG Telomere Length Assay (Roche, Germany) kit. The product derived from the

telomere PCR assay was normalized with the product obtained by the β-globin PCR assay and telomere length was measured in base pairs (bp). The primers used in amplifications were described in table1.

Cycling conditions (for both telomere and HBG amplicons) are: 10 min at 95°C, followed by 40 cycles of 95°C for 15 sec, 60°C for 1 min, followed by a meltingcurve analysis.

A plot of CT (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) was generated by performing serial dilutions of the standards ranging between 10^{-1} to 10^{-6} dilution. This linear curve allows absolute quantitation of unknowns in the same PCR run.

Statistical analysis:
Collected data were analyzed using SPSS 16 (SpssInc, Chicago, ILL Company). Categorical data were presented as number and percentages while quantitative data were expressed as mean ± standard deviation, and range. Chi square test (X2) test was used to analyze categorical variables. Quantitative data were tested for normality using KolomogrovSmirnove test, using Wilcoxon test, Man Whitney U test and Spearman's correlation coefficient (rho) as proved to be non-parametric. P<0.05 was considered significant.

Table (1): Primer sequences in amplification reaction **[13]**

Gene	Primers	
	Forward	**Reverse**
THR[*]	5'GGTTTTTGAGGGTGAGGGTGAGGGTGAGGGT GAGGGT-3'	5'TCCCGACTATCCCTATCCCTATCCCTATCCC TATCCCTA-3'
HBG[**]	5'-GCTTCTGACACAACTGTGTTCACTAGC-3'	5'-CACCAACTTCATCCACGTTCACC-3'

THR[*]telomere hexamer repeats.
HBG[**] human β-globin.

RESULTS

This cohort prospective study was conducted on 25 *H. pylori*-positive patients (16 males and 9 females) with mean age 39.4±8.12 years and 17 H. pylori- negative patients served as a control group (12 males and 5 females) with mean age 38.5±8.5 years. There was insignificant statistical difference between patients and controls as regard sex and age, (P value = 0.65 and 0.74 respectively) (Table 2).

The absolute TLs was shorter in *H. pylori* positive patients than control group (*H. pylori* negative patients) before treatment regimen (2326.4 ± 165.06 bp Vs 3046.7 ± 331.60 bp respectively) with highly statistically significant difference between both groups (P<0.001) (Table 3).

In Table (4) there was highly statistically significant difference between *H. pylori* positive patients before and after eradication therapy (P<0.001). The telomere length was significantly increased after eradication therapy (absolute TLs was 2326.4±165.06 bp before treatment Vs 2842.3 ± 296.04 bp after treatment).

There was insignificant difference in absolute TLs in *H. pylori* positive patients after eradication therapy compared to control group (P = 0.053) (Table 5).

There was significant negative correlation between ages of patients and TLs before and after treatment (before treatment, r= -0.713, P<0.001, after treatment r= -0.846, P<0.001) (Figs. 1,2 respectively). While as regard the relation between sex and TLs there was no significant relation (before treatment TLs in males was 2335.5 ± 166.97 bp, in females it was 2310.1 ± 170.28 bp and P value = 0.51 and after eradication therapy TLs was in males 2881.6 ± 305.00 bp and in females it was 2772.3 ± 282.65 bp with P value = 0.55) (Table 6).

Table (2): Comparison between studied groups regarding sex and age

Variable	Group I (N = 17) *H. pylori* negative cases	Group II (N = 25) *H. pylori* positive cases	Test	P value
Sex Females (%) Males (%)	5 (29.4) 12 (70.6)	9 (36.0) 16 (64.0)	X^2=198	0.65
Age (years) Mean ±SD; (range)	38.5 ± 8.5 (25-52)	39.4 ± 8.12 (25-52)	t= 0.32	0.74

Table (3): Comparison between *H. pylori* positive patients and control group as regard TLs before treatment

Study groups	TLs*in bp
	Mean ±SD
H. pylori **positive patients before treatment (No.=25)	2326.4±165.06
H. pylori **i negative control group (No.=17)	3046.7±331.60
Test	***MWU Z= 5.25
P value	**< 0.001**

*Telomere length, **Helicobacter pylori*, ***MWU= Man Whitney U Test

Table (4): TLs in *H. pylori* positive patients before and after eradication therapy

Study groups (No.=25)	TLs*in bp
	Mean ±SD
*H. pylori** positive patients before eradication therapy	2326.4±165.06
*H. pylori** positive patients after eradication therapy	2842.3±296.04
Test	Wilcoxon test Z= 4.4
P value	**< 0.001**

*Telomere length, **Helicobacter pylori*.

Table (5): Comparison between *H. pylori* positive patients and control group as regard TLs after eradication therapy

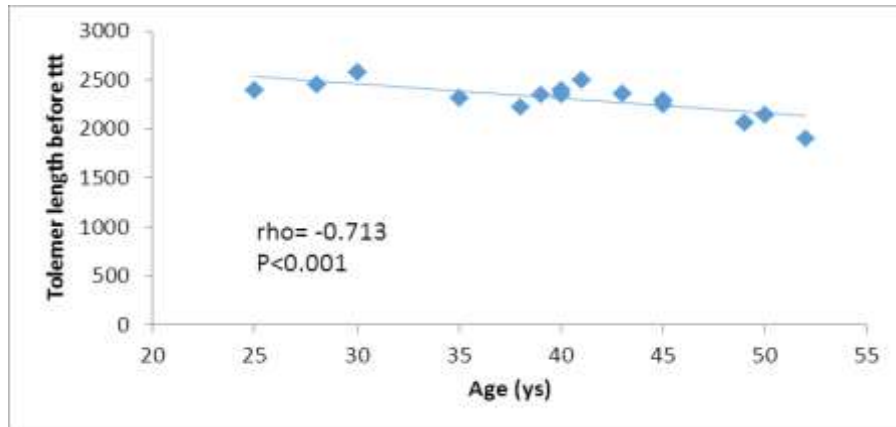| Study groups	TLs*
	Mean ±SD
H. pylori ** positive patients after eradication therapy (No.=25)	2842.4±296.04
*H. pylori** negative control group (No.=17)	3046.7±331.60
test	MWU Z = 1.93
P value	0.053

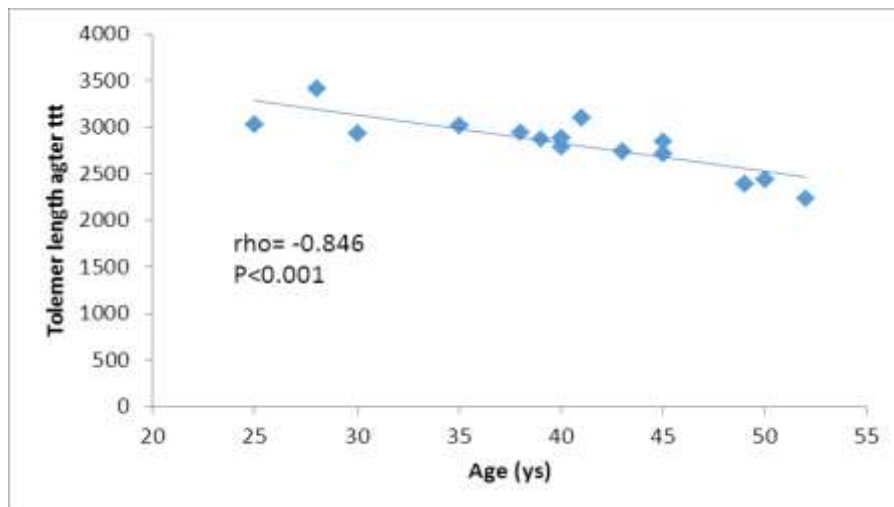**Figure (1):** Correlation between age and telomere length before treatment

Figure (2): Correlation between age and telomere length after treatment

Table (6): Relation between sex and telomere length

Sex	Telomere length (bp) Mean ± SD	Test	P value
Before eradication therapy			
Male (N = 16)	2335.5 ± 166.97	MWU	0.51
Female (N = 9)	2310.1 ± 170.28	Z = 0.65	
After eradication therapy			
Male (N = 16)	2881.6 ± 305.00	MWU	0.55
Female (N = 9)	2772.3 ± 282.65	Z = 0.59	

DISCUSSION

Telomeres are special chromatin structures that protect the ends of chromosomes from degrading and restructuring activities [4]. 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 [5]. 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 [14]. Inflammation, oxidative stress and increased cell replication are major environmental factors associated with accelerated shortening of telomeres [15]. Meta-analyses suggest 1.4 to 3.0 fold increased risk of cancer for those with the shortest versus longest telomeres [16,5]. *H. pylori* infection in gastric mucosa leads to chronic inflammation and complications, such as ulcer, metaplasia and cancer. Even though the relation of *H. pylori* and the pathogenesis of gastric cancer is not completely known, it has been shown that a change in telomere length and telomerase activity in pre-neoplastic and cancerous tissue might arise. This change is considered a stage or cause in the development of cancer [17]. Pre-neoplastic changes that might progress into gastric cancer are found in around 50% of people infected with *H. pylori* and TL has been found to be shorter in some tumors a scolon cancer and gastric cancer compared to normal tissue [8,9]. This study was performed to assess the absolute TLs in gastric mucosa of *H. pylori* infected patients compared to *H. pylori* negative controls and determine the effect of eradication of *H. pylori* infection on TLs. In the present study we found highly significant shortening in TLs in *H. pylori* positive patients than control group (P<0.001), this finding was in agreement with Kuniyasu et al. [18]. Who reported that TL was significantly shorter in the group infected by *H. pylori* than in the uninfected group, in the same hand Aida et al. [19] demonstrated that *H. pylori*–positive gastric mucosa has been shown to have shorter TL than H. pylori negative mucosa. Similarly Aslan et al. [20] found that the telomere length was found to be 2958.9±1345.7 bp in the control group and 2481.2±1823bp in the study group prior to eradication; however, this difference was not significant (p=0.11). In the present work, after *H. pylori* eradication therapy, there was highly statistically significant elongation in TLs in *H. pylori* positive cases (P<0.001), that in agreement with Aslan et al. [20] who found that The telomere length was found to be 3766.3± 1608.8 bp in the study group after eradication. In this group, the telomere length was significantly increased after eradication compared to the period before eradication (p=0.01), and considered this finding provides new approach for effectiveness of *H. pylori* eradication therapy in increasing the TLs and decrease incidence of gastric cancer, also Chung et al. [5] 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. This finding was considered to provide the effectiveness of *H. pylori* eradication therapy in increasing the TLs and decrease incidence of gastric cancer. Also in the present study we found significant negative correlation between age and absolute TLs in patients group before and after treatment (P<0.001), this result disagreement with Aslan et al. [20] who found negative correlation but insignificant (r = 0.172, p>0.05), while Hou et al. [21] reported that telomeres were significantly shorter in association with aging (P<0.001), and 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. As regard relation between TLs and sex the present work found insignificant relation between TLs and gender before eradication therapy in patients group (2335.5 ± 166.97 bp in males and 2310.1 ± 170.28 bp in female with P value 0.51) this in agreement with Aslan et al. [21] as they found TLs before eradication was found (2721.3 ± 2003.44 bp in males, 2161.1 ± 1608.6 bp in females, p>0.05), on the same hand Hou et al. [21] reported insignificant relation between TLs and sex (P value = 0.09), when studying TLs in gastric cancer related to *H. pylori* positivity.

CONCLUSION

H. pylori positive patients have significantly shorter absolute TLs in their gastric mucosa compared to *H. pylori* negative controls. *H. pylori* eradication therapy increases the absolute TL in *H. pylori* infected patients and that can open new approach for consider effectiveness of H. pylori eradication therapy in increasing the telomere length one of preventable methods for gastric cancer.

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