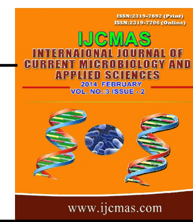

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Original Research Article

Tip-alpha gene expression of *Helicobacter pylori* using Real Time RT PCR and association with clinical outcomes

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A B S T R A C T

The Tip-α gene is related to carcinogenesis of *Helicobacter pylori*. Carcinogenesis of Tip-α protein has proven in vitro but not in vivo. There are not enough reports considering level of tip-α gene expression in *Helicobacter pylori* strains isolated from patient with different clinical manifestations. The aim of this study was to evaluate tip-α gene expression in *Helicobacter pylori* strains isolated from Iranian patients with Non Ulcer Dyspepsia (NUD) and Gastric Cancer (GC). We measured the tip-α gene expression by Real-Time PCR in patients with different clinical outcomes. Real-Time PCR results showed the tip-α gene expression in *H. pylori* strains from patients with Gastric Cancer (subject samples) was 1.34 times higher in compare to strains isolated from patents with Non Ulcer Dyspepsia (Calibrator samples) . However there was not significance relationship between clinical outcomes and amount of tip-α gene expression in *H.pylori* strains isolated from Iranian patients. This study suggests a protocol for bacterial gene expression survey from biopsy without culture and closed to *in vivo* situation.

Keywords

Helicobacter pylori; tip-α gene expression; Real-Time RT PCR; Clinical outcomes.

Introduction

Helicobacter pylori (*H. pylori*) is a gram-negative, spiral and motion bacterium that has infected nearly half of the world's population. Human is known as the host (1). *H. pylori* after transportation through the fecal-oral, take up residence in the mucus layer overlying the gastric epithelium. Several pathogenesis factors have been identified in this bacterium such as cag that can be related to the pathogenesis of toxin production (cag PAI) and vaculation cytotoxin (Vac) and the urease enzyme. Pathogenesis of these factors is well proven in the vitro conditions (2;3; 1). Numerous studies have been established the relationship of virulence factors vacA, Cag PAI and urease with gastric cancer. (1;) In recent years, a group of genes that encode proteins associated with the pathogenesis

of *H.pylori* are found be associated with cancer. Of This genes family, the Tipα (Tumor Necroting Factor- alpha Inducing-proteins) which includes: the Tip-α protein coding genes in strains 26695 (HPO596), HP-MP1 protein coding genes in strains SR779 and possibly C) JhpO 543 gene in strain J99 (1) has gotten very interest. There is a homology in sequence of 20 amino acids at N terminal among them. Tip-α is a 19 to 21.8 kDa protein that includes of two monomers 19 KD and 38 KD. The activation form of this protein is a homodimer that composed of 192 amino acids (4;5;6;1). Tip-α protein increases activation of Nuclear Factor Kappa-B (NF-KB) caused to more expression of genes IL-1, IL-8, TNF-α in host cells. TNF-α protein inhibits apoptosis and stimulates cell growth in the carcinogenesis pathway. Carcinogenic aspects of Tip-α protein is special in among other carcinogenic agents (7;8; 9;1;10). Several characters of the Tip-α protein that has been identified are including: the homodimer structure and independent secretion of the type IV system, NF-KB activation is independent of PAI Cag pathway, there is protein in cytosol and core of infected cells, using Biacore measurement (Pull down and surface Plosmonresonans) binding activity of this protein to DNA has been demonstrated in In vitro and this protein is immunogenic highly (3;5;). Primary structure of Tip-α protein is monomer that outside of cytosol will be biological activation dimmer. This protein binds to DNA after entering into the host cell nucleus and active NF-KB. Even HTLV1 as a viral oncoproteincan not bound to host cell DNA. *Tipα* gene locus is located outside of other pathogenesis areas such as Vac, Cag PAI and urease gene in *Helicobacter pylori* (1). Recent studies show that Tipα protein can creates cancer in Bhas 42 (cells in BALB/T3T that have been infected by V-Ha-vas) and increase gene expression of Ras (growth and cell division factors) in these cells. This increased expression occurs rarely in normal cells. Carcinogenic potential of Tip-α protein hase proven in stomach cells of rat (MGT-40) (2;3;10). Some studies show different of *tip-α* gene expression in isolated strains of *H. pylori* from Gastric cancer and NUD biopsies (1;10).In published studies, gene expression survey of *tip-α* was performed after culture of *H. pylori* from gastric biopsy samples (1;10). If there was any effect on *tip-α* gene expression in the gastric conditions, these effects should be considered after culture. So in present study, for assessment level of *tip-α* gene expression closed to in vivo situation, bacterial RNA directly was extracted from biopsy after transmission of biopsies into RNAlater and then a Single-Primary PCR was performed for increase of amount of cDNA.

Materials and Methods

Patients and Samples

Gastric biopsy specimens were collected from patients undergoing gastric endoscopy referring to the Imam Khomeini hospital and Dey Hospital in Tehran in duration of 8 months in 2013. Seventy five samples from patients with NUD as calibrator (25 patients, mean age of 48) and 75 specimens from patients with gastric cancer as subject (25 patients, mean age 61 years) were collected. Inclusion criteria included patients with *H. pylori* infection proven by the rapid urease test of *H. pylori*, patients with an absence of non-gastrointestinal chronic medical conditions and the absence of contraindications to upper gastrointestinal endoscopy, patients providing informed

consent, and patients with a willingness to complete a standardized data-collection form. Three antral biopsies were taken from each patient during routine Endoscopy by special physician. The biopsies were immediately stored in RNAlater reagent (Qiagen Company) at -20°C until RNA extraction. State on age, gender, smoking and drinking for all of patients was recorded.

RNA extraction and Reverse Transcriptase (RT-PCR)

Total RNA was extracted by Tripure reagent (Roche Company) according to the manufacturer protocol. The RNA concentration was measured by OD at 260 nm and its integrity was confirmed by electrophoresis on %1 agarose gel. Extracted RNA was stored at -80°C for using of Reverse transcription in next step (11) CDNA was synthesized from total RNA (5µg) by Quantitect Reverse Transcription (Qiagen Company), according to manufacturer instructions. The integrity of cDNA was tested by PCR assay of the ureA gene and visualized on %2 agarose gel after electrophoresis. Briefly for synthesize of cDNA, RNA (10 µl), primer (1 µl), gDNA (2 µl) and RNase free water (2 µl) mixed and then incubated in 42°c for 2 min. Superscript III (1µl), RT buffer (4µl), RT primer (1µl) mixed with mastermix, Then incubated in 42 °C for 15 min, Finally incubated in 95°C for 3 min cDNA stored in -20°C for later using.

Primer designing

Primers were designed for Primary-Single PCR and Real-Time PCR by the Primer3 software then were blasted with online NCBI blast program. Primers were synthesized by MWG Company (USA). The sequences of designed primer are listed in Table 1.

Primary Single-PCR

A primary Single-PCR was performed for increase of little amount of bacterial cDNA. Real-Time PCR was performed on diluted products of primary Single-PCR. 5λ of cDNA was used for PCR. Program of PCR was 95°C for 1 min, 15 s at 95°C (Denaturation step), 55°C for 15 s (Annealing step), 72°C for 30 s (Extension step) and final extension step for 7 min. After PCR amplification, PCR product was observed in %1.5 agarose gel. Then serial dilutions of the PCR product were allocated for using in Real-Time PCR.

Relative Real Time PCR

Amplification reactions were performed on a Corbett Rotor-Gene 6000 Real-Time PCR System based on the SYBR Green methodology. Each Microtub were contained diluted cDNA (2µl) , Tip-alpha and ureA specific primers (2µl), SYBR® Green with Rox (10µl) and RNase free water (6µl) (Qiagen Company). Relative amount of tip-α cDNA was measured by Real-Time PCR.

Thermal cycling condition was: 10 min at 95°C for initial activation, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. The Calibrators (NUD) and subject samples (GC) were assayed in triplicate on each 36-well. The ureA as housekeeping gene was evaluated in subject and Calibrators because expression of this gene showed the lowest variation in *H.pylori*. The relative quantification survey of *tip-α* gene was calculated in relative with ureA gene by Rotor-Gene 6000.

Table.1 Sequence of primers for Primary-Single PCR and Real-Time PCR

Gene	Forward Primers	Reveres Primers
tip-α	5´-TGAGGAAGTGTTTGCGTTAGTGCG-3´	5´-TAGCCACAAGGATACTCACCGCTT-3´
ureA	5´-TGCGCCCTTCTTGCATCAATTCAG -3´	5´- TGCTCCACTATGCTGGAGAATTGG-3´

Statistical analysis

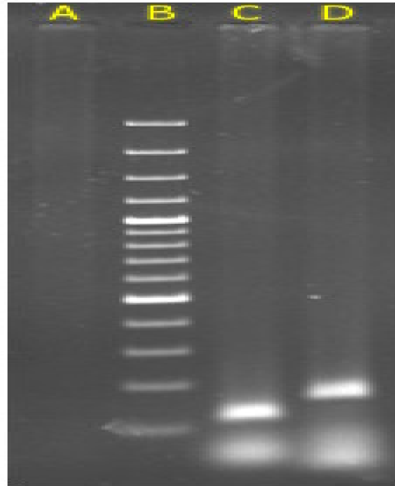
The relative expression of tip-alpha gene was reported by the Mean as a point estimator and the range of values by ct. (12;13;14;15). ΔΔCt method was used for comparison of *tip-α* gene expression in Calibrator and subject groups (16;13;14;17;18). By using of SPSS software, the Student's t-test used to compare significance of *tip-α* gene expression different between groups. A significance level of 0.05 ($P< 0.05$) was considered.

Results and Discussion

A primary Single-PCR was performed for all of cDNA samples. The Primers of *tip-α* and *ureA* genes were good because was not found any nonspecific bands (Figure1). The products of primary Single-PCR were allocated and were diluted in the equal conditions for using in Real-Time PCR step. Real-time PCR analysis was performed for all of 50 samples (25 calibrator samples and 25 subject samples). PCR Efficiency of the samples was determined by dilution series of cDNA (Corbett Machine, Rotor-Gene 6000). Range of variation in PCR Efficiency for *tip-α* and *ureA* genes was 0.82 – 1.36. Any sample that had PCR Efficiency out of standard range was emitted from our study (Figure2). In the subject group, ct mean of ureA gene and *tip-α* gene was detected 12.95 and 18.6, respectively. In the calibrator group, Ct mean of ureA gene and *tip-α* gene was detected 13.32 and 19.4, respectively. After normalization with the urea reference gene and comparison with the respective adjacent NUD mucosa, the relative expression of *tip-α* gene in the GC group was found to be increased in 1.34 times in compare with NUD group (ΔΔCt method). Considering all samples, the ΔCt mean ± SD gene expression levels in the calibrator and subject groups were 5.72 ± 2.42 and 5.10 ± 3.52, respectively (Figure 3). So no significant different in *tip-α* gene expression was founded between two groups ($P< 0.05$).

In the research of Masami et al (1;10), *tip-α* gene expression in patients with GC and NUD was surveyed. They showed expression of *tip-α* gene in *H. pylori* strains isolated from GC biopsy is different in compare with *H. pylori* strains isolated from NUD biopsy. They cultured bacteria from biopsy because trace amount of extractionable bacterial genome in gastric biopsy specimens. It is could be mentioned, amounts of bacterial genome and bacterial gene expression change after culture of bacteria relation to situation of theme in stomach. Accordingly we tried to keep and fix amounts of bacterial RNA close to *in vivo* situation by using of RNAlater (Roche). We designed a primary Single-PCR by specific primers for increase of *H. pylori* cDNA. While the same other studies have used culture step for increase of *H. pylori* genome (1;10).

Figure.1 The bands of Primary single-PCR products on %1 agarose gel electrophoresis.



Fragment size for *ureA* gene and *tip-α* gene was 130 bp and 185 bp respectively. A row is negative sample, B row is 100 base/pair ladder, C row is *ureA* gene sample and D row is *tip-α* gene sample

Figure.2 PCR Efficiency: Corbett machine calculate PCR Efficiency in end of any reaction automatically. PCR Efficiency is necessary for verification of PCR reactions.

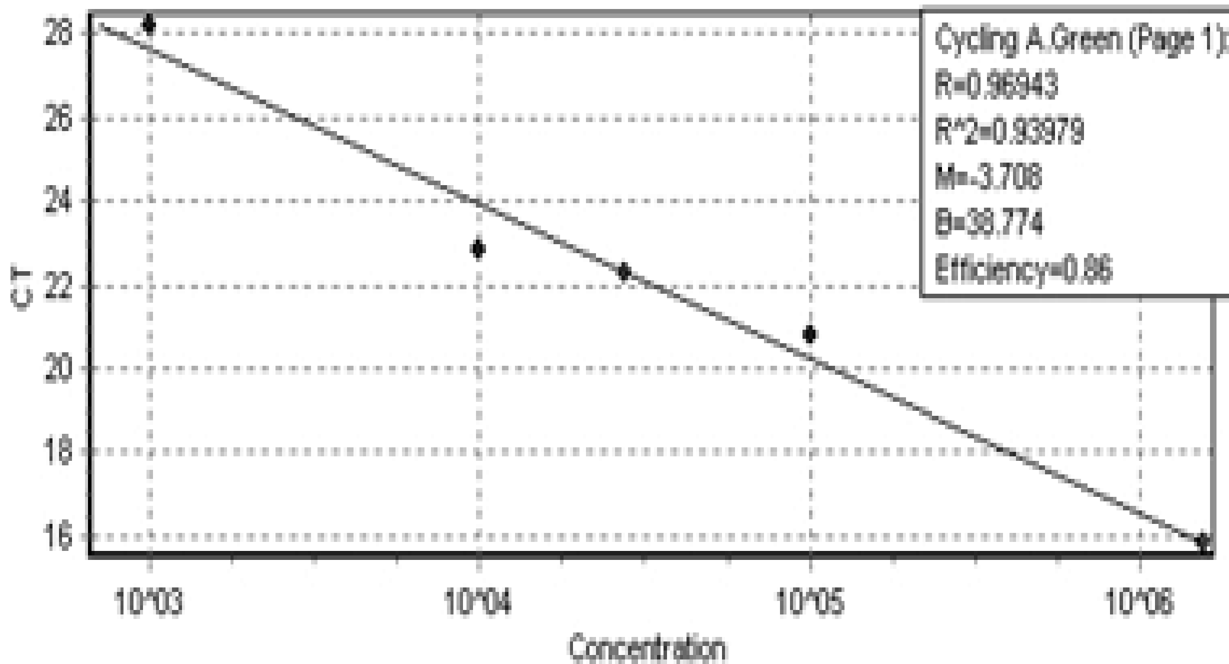
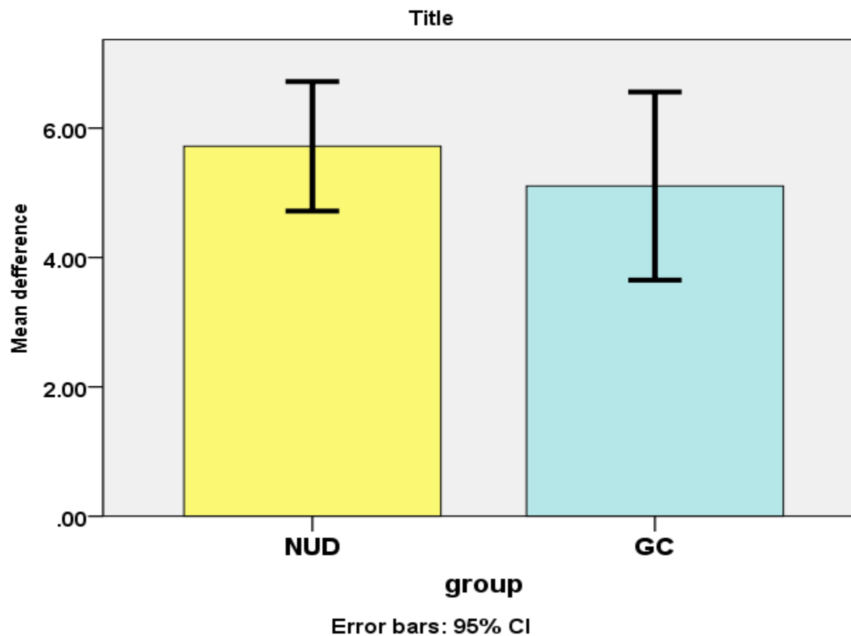


Figure.3 Comparison of ΔCt means between NUD group and GC group



Bacterial RNA directly was extracted from biopsy specimens by Tripure kit (Roche Company). The results of the Primary Single-PCR were good for increase of low amounts of *H. pylori* cDNA. We used equal amounts of cDNA that produced in duration of Primary Single-PCR for Real-Time PCR. The tip-α gene expression in subject group was 1.34 times calibrator group that was not significant. We performed a primary Single-PCR by specific primers for increase of low amounts of bacterial genome in biopsy specimens.

Advantage of this method is increasing bacterial genome amount in an identical ratio to in vivo situation without using of culture step. The suggestion is, if *tip-α* gene expression is variable in different samples (GC vs. NUD) and in different conditions (In vivo vs. In vitro) why not use from its promoter as a condition promoter, a promoter for regulation of gene expression that will be regulated by environmental PH factor. Although more research is need in this regard.

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