Zak Gene Expression in Patients With *Helicobacter Pylori* Infection

Delniya Khani

Kurdistan University of Medical Science: Kurdistan University of Medical Sciences

Manouchehr Ahmadi Hedayati (✉ dr.ahmadi2000@gmail.com)

Kurdistan University of Medical Sciences <https://orcid.org/0000-0003-0654-8918>

Farshad Sheikhesmaeili

Kurdistan University of Medical Science: Kurdistan University of Medical Sciences

Roghaie Ghadiany

Kurdistan University of Medical Science: Kurdistan University of Medical Sciences

Sherko Nasser

Kurdistan University of Medical Science: Kurdistan University of Medical Sciences

Original Research

Keywords: zak, *Helicobacter pylori*, Gastritis, Gastric cancer, gene expression, Gastric Antral epithelial cells

Posted Date: February 16th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-195060/v1>

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Version of Record: A version of this preprint was published at Journal of Gastrointestinal Cancer on February 23rd, 2021. See the published version at <https://doi.org/10.1007/s12029-021-00611-3>.

Abstract

Background: ZAK protein is a member of the MLK family proteins defined as mediators in the cell cycle. A survey of *zak* gene expression in gastric antral epithelial cells (GAECs) of gastritis and gastric adenocarcinoma patients with *Helicobacter pylori* genotypes infection can elucidate carcinogenesis of *H. pylori* genotypes.

Methods: In a case-control study, *zak* gene expression was evaluated in GAECs biopsy samples of gastritis patients with (n 23) and without *H. pylori* infection (n 27) and gastric adenocarcinoma patients with (n 21) and without (n 32) *H. pylori* infection. The *zak* gene expression was measured using the relative Real-Time RT PCR.

Results: *zak* gene expression was higher in GAECs of gastritis cancer than in gastric adenocarcinoma, indicating the protective effect of ZAK against gastric cancer ($p < 0.005$).

Conclusion: Reducing *zak* gene expression has the negative correlations with *H. pylori* infection and gastric adenocarcinoma.

Introduction

Helicobacter pylori is a gram-negative bacterium that has been colonized in the gastric pylor area of more than half the world's population (1). In developing countries, the prevalence of *H. pylori* infection is up to 80%, while it is less than 30% in developed countries (1). The prevalence of *H. pylori* infection in Iran, as a developing country in the west of asia, has been reported between 36% to 90% (1). The *H. pylori* persistent colonization in infected people lead to inflammation and gastrointestinal diseases (2-3). *H. pylori* pathogenesis is attributed to virulence factors including CagA, VacA, and surface adhesins (3-4). *H. pylori* binds to the surface of gastric antral epithelial cells (GAECs) and injects CagA protein, activating the mitogen-activating protein kinase (MAPK) signaling (5-6).

MAPK is a member of the serine/threonine kinase family, activated by a rang of proteins, including extracellular signal-regulated kinases (ERK1/2), Jun N-terminal kinase (c-JNK), and P38 (6-7). The MAPK signaling pathway plays a vital role as a mediator in cell physiological events, including mitosis, motility, adhesion, metabolism, and apoptosis (8). Besides, MAPK members contribute in cell signal cascades during infection (6). The imbalance between *H. pylori* infection and MAPK signaling in GAECs conduct fate of infection to proliferation or apoptosis. In contrast, a balance leads to symbiosis and long survival of the pathogen (5-9).

MLKs are a family of serine/threonine protein kinases whose is mediated by mitogen activating protein kinases (MAPKKs) (10). The set of MLKs is divided into three groups based on sequence similarity in the catalytic domain, including MLKs, leucine zipper containing kinase AZK (ZAK), and sterile- alpha-motif kinase (7, 10-11).

Zak [mitogen-activated protein kinase kinase kinase 20 (MAP3K20)] has two isoforms, α and β , as a critical regulator of the MAPK pathway that conduct cell survival and the inflammatory response (12-13). Zak is a mediator in oxidative stress as a pro-apoptotic factor, positive regulator of epidermal growth factor (EGFR), regulator of WNT signaling pathways, and finally a proliferation signaling factor in adenocarcinoma cells (14-15). Previous studies report that the mixed-lineage kinases (MLK) modulate proliferation in gastric adenocarcinoma cells (16-17). As a contradictory finding, recent Zak protein studies suggest an antitumor impact on gastric adenocarcinoma (7, 10-12, 16). This is a confusing finding that results of other studies indicate *zak* gene overexpression in the other types of adenocarcinomas, including the stomach, breast, bladder, and colorectal (13). A study on the EGFR pathway showed that both *zak* isoforms regulate adenocarcinoma cell proliferation in a kinase-dependent manner (17). Decreased activity of ZAK protein reduces Hela and HCT116 cells' motility, and increases expression of the MAPKs genes, including JNK and ERK (18). Since JNK regulates cellular processes including inflammation and apoptosis, and ERK is usually activated in response to various mitogens, ZAK can be considered as the confocal key to determine cell fate (18).

The previous studies show a high prevalence of *H. pylori* infection and gastric adenocarcinoma in Iran. This study aimed to survey *zak* gene expression in GAECs of gastric adenocarcinoma and gastritis patients with *H. pylori* infection.

Methods

A case-control study was designed base on statistical protocols. The gastric antrum biopsy specimens were obtained from gastritis and gastric adenocarcinoma patients referred to Tohid and Shahid Ghazi hospitals in Sanandaj city, in Kurdistan province in the west of Iran. The sample required number was calculated using Cochran's formula based on the prevalence of gastric carcinoma and *H. pylori* infection in Kurdistan province (19-21). 50 gastric adenocarcinoma and 53 gastritis patients enrolled in study. Patients treated with anti-*H. Pylori* antibiotics, alcohol consumption, and smoking were excluded from the study. The gastric antral biopsy specimens were collected in a sequential and available sampling process by a gastroenterologist for 18 months from September 2018 to March 2019. The urea breath test was used to find active *H. pylori* infection. In order to molecular assays, the biopsy specimens were immediately dropped into RNALater solution and transferred to the Molecular Microbiology research laboratory in Kurdistan University of Medical Sciences. To evaluate the pathological assays, pathologic biopsy specimens were collected from the tumor area of gastric adenocarcinoma patients and transferred to the pathology laboratory of Tohid Hospital

in Sanandaj city. Pathological gastric adenocarcinoma specimens were from four areas involved in gastric cancer, including the cardia, pylorus, body, and Lesser Curvature. By using Takara kit, total RNA, including microbes and GAEs RNA, was extracted from patients' gastric antral biopsy specimens, then cDNAs were synthesized. The *H. pylori* cDNA was determined using specific *vacA* m2 primers and PCR method, then the PCR product was confirmed by sequencing (accession number MK642592.1). By using specific primers of *H. pylori* virulence genes (Table 1) and the PCR method, *H. pylori* virulence genes' cDNA in gastric biopsy specimens of patients with *H. pylori* infection was determined.

Table 1. Primers used in this study.

Specific Primers	Sequence	Annealing Tm °C	Product Size bp	Reference
16s rDNA <i>H. pylori</i> F 16s rDNA <i>H. pylori</i> R	CTGGAGAGACTAAGCCCTCC AGGATCAAGGTTTAAGGATT	50	446	This Study
<i>cagA</i> F <i>cagA</i> R	TGACCAACAACCACAAACCG TCAGGATCGTATGAAGCGACAG	57	108	This Study
<i>cagA</i> EPIYA-C F <i>cagA</i> EPIYA-C R	AAGAAAGCAGGACAAGCAGC CTAACCGATCGCCCTACCTT	55	188	This Study
<i>cagT</i> F <i>cagT</i> R	AGGGTGTGGTGTATGATAGCG TGCTTGTTGTTTGCTCCACT	55	154	This Study
<i>cagE</i> F <i>cagE</i> R	GAATGGAGCGAGCGATGAAA TAGGAATTTGCAGCGCTCAC	56	163	This Study
<i>cagY</i> F <i>cagY</i> R	AGTTCAAGTGGCGCTAGATTG ACAAGCCTTTCAAGCATTCTG	57	200	This Study
<i>vacA</i> s1/s2 F <i>vacA</i> s1/s2 R	ATGGAAATACAACAAACACAC CTGCTTGAATGCGCCAAAC	55	259/286	Atherton et al. (22)
<i>vacA</i> m1/m2 F <i>vacA</i> m1/m2 R	TGGATAGTGC GACTGGGTTT TCCATGCGGTTGTTGTTGTT	54	205/220	This Study
<i>iceA1</i> F <i>iceA1</i> R	GTGTTTTTAACCAAAGTATC CTATAGCCASTYTCTTTGCA	45	247	van Doorn et al. (23)
<i>iceA2</i> F <i>iceA2</i> R	GTTGGGTATATCACAATTTAT TTRCCCTATTTTCTAGTAGGT	47	229/234	van Doorn et al. (23)
<i>hopQI</i> F <i>hopQI</i> R	ACGAACGCGCAAAAACCTTTA TTGCCATTCTCATCGGTGTA	55	187	Sicinschi et al. (24)
<i>hopQII</i> F <i>hopQII</i> R	ACAGCCACTCCAATCCAGAA AACCCACCGTGGATTTTAG	55	160	Sicinschi et al. (24)
<i>babA2</i> F <i>babA2</i> R	CAATGCGGTGCGTGAAAATC ATACCCTGGCTCGTTGTTGA	57	205	This Study
<i>babB</i> F <i>babB</i> R	CAATCCCCGGCGTATCAAG ATTGCAAGTGATGGTCGTCG	56	175	This Study
<i>sabA</i> F <i>sabA</i> R	TCTCTCGCTTGCGGTATCAT AGCTCAATGTTGTTGGCGTT	56	204	This Study
<i>sabB</i> F <i>sabB</i> R	GCATTCAAACGGCGAACAAC TCCTGTGCAGTTCCCATCTT	56	248	This Study
<i>alpA</i> F <i>alpA</i> R	CGCTCCTATCAAAACCGCTC TTCCCGTCCAACCTACCGAA	55	185	This Study
<i>alpB</i> F <i>alpB</i> R	TCAACTTGCGAGCAGACCTA AGCCATAGACCCCATACAG	57	218	This Study
<i>oipA</i> F <i>oipA</i> R	CTCCACGCTGAAAGGAATGG CCATTTCCTGCGAATCGGTT	55	233	This Study

*The primers yield a fragment of 229 or 334 bp depending on the presence of a repetitive sequence of 105 nucleotides in some iceA2 alleles [40].

The *zak* gene expression in gastric antral biopsy specimens of gastritis and gastric adenocarcinoma patients with and without *H. pylori* was surveyed by using the Real-Time RT PCR method (25). The PCR primer sequences used were as follows: *zak* sense primer 5ACTTTGGTGCCTCTCGGT3 and antisense primer 5TTCTGGGGCAACTGCTT3 (product size 249 bp); GAPDH sense primer 5GAAGGTGAAGTCCGAGTCAAC3 and antisense primer 5CAGAGTTAAAAGCAGCCCTGGT3 (product size 71 bp) (7). The PCR amplification began with a 10 minutes denaturation step at 95°C and then 40 cycles of denaturation at 95°C for 15 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 60 seconds (7). The relative amounts of the *zak* expression gene between gastritis (control group) and gastric adenocarcinoma (case group) patients were surveyed by ΔCt (threshold cycle) = Ct (ZAK) Ct (GAPDH). The ratio of *zak* mRNA copies to GAPDH was then calculated by $2^{-\Delta Ct}$ (7, 25).

Statistical methods:

The relationship between patients' qualitative demographic variables including sexuality and *H. pylori* infection was examined by Chi-square tests, including Fisher's exact (21). At first, the frequency distribution of *zak* gene expression in patients' GAECs was computed by using the Kolmogorov-Smirnov statistical test (21). Correlation of *zak* gene expression with *H. pylori* virulence genes cDNA and clinical futures, including disease, tumor grade, and gastric adenocarcinoma tumor area, was evalutaed by using Pearson and Spearman statistical methods. In consequence, Spearman test was used to assess the statistical relationship between abnormal distribution of *zak* gene ΔCt s with patients' demographic variables (21). The statistical relationship between *zak* gene expression and the *H. pylori* virulence genes cDNAs in patients with and without *H. pylori* infection was surveyed by using Mann-Whitney, and Kruskal-Wallis (21). A nonlinear regression plot was designed for the relationship between the age of patients and *zak* gene ΔCt s.

Results

Table 2 shows the patients' demographic results. No significant relationship between *H. pylori* infection and clinical disease was found ($p > 0.05$). Similar to relevant previous studies' results, the results showed that gastric cancer's risk gradually increases with age increasing ($p < 0.001$). The prevalence of gastric cancer among men and women is correlated to society's cultural and economic level. Our study showed that the prevalence of gastric cancer in men in Kurdistan province, in the west of Iran, is more than twice that of women ($p < 0.05$). 61% of men and 61.9% of women in this study had an active *H. pylori* infection, so Fisher's exact test results found no significant relationship between *H. pylori* infection and patients' gender (Table 3). The *H. pylori* infection highest and lowest rates were among people aged 31 to 45 (57.89%) and 76 to 85 (11.11%), respectively ($p < 0.01$).

Table 2
Demographic characteristics of patients and correlation of demographic variables

	Gastritis	Gastric cancer	Total	P-value
<i>H. pylori</i> infection (Positive/Negative)	23/27	21/32	44/59	0.513
Sex (Male/Female)	24/26	37/16	50/53	0.024
Age group (18–30/31–45/46–60/61–75/76–85)	10/18/17/5/0	0/1/14/29/9	10/19/31/34/9	0.000

Table 3
Demographic characteristics of patients with *H. pylori* infection

<i>H. pylori</i> infection (n: 103)	Sex (n)		Age Group (n)				
	Male	Female	18–30	31–45	46–60	61–75	76–85
<i>H. pylori</i> infection (Positive)	28	16	2	11	14	16	1
<i>H. pylori</i> infection (Negative)	33	26	8	8	17	18	8
Total	61	42	10	19	31	44	9

The results of *zak* gene expression in patients' GAECs showed that the variables of age, type of disease, *H. pylori* infection, and gastric adenocarcinoma tumor grade have correlations with *zak* gene expression (Table 4). Although a statistically significant relationship was observed between *zak* gene expression and tumor grade, this statistically significant relationship was not observed in the tumor area. (Table 4) ($p < 0.05$).

Table 4
Correlation of *zak* gene expression in gastric antral epithelial cells of patients with gastritis and gastric adenocarcinoma with patients' demographic characteristics Spearman statistical test.

	Sex	Age Group	Disease	<i>H. pylori</i> infection	Gastric cancer Area	Tumor Grade
Zak Correlation	0.129	0.210	0.305	-0.329	-0.170	0.532
P-value	0.192	0.033	0.002	0.001	0.450	0.011

The highest rate in *zak* gene expression was observed in GAECs infected with *H. pylori vacA s1m2* genotype ($p < 0.01$) (Table 5). The results of this study showed that *H. pylori* infection correlates with *zak* Δ Cts and fold changes in *H. pylori* positive-samples is up to 2.59 times ($p < 0.005$) (Table 6). Based on $\Delta\Delta$ Ct formula results, the *zak* gene expression was higher in gastric antral biopsy specimens of gastritis patients than gastric adenocarcinoma patients, This findings indicate to the protective effect of *zak* gene expression against gastric cancer ($p < 0.005$). The *zak* gene expression also gradually decreased with increasing tumor grade, Therefore in biopsy samples with tumor grade 3, the lowest *zak* gene expression was observed. In this study, grade 4 disease was not studied, and comparison was performed among grade 1, 2, and 3 tumor grades.

Table 5
Correlation between *zak* gene expression in gastric antral epithelial cells of gastritis and gastric adenocarcinoma patients with *H. pylori* virulence genes cDNAs using Spearman test.

	<i>vacA s1m1/s1m2</i>	<i>cagA</i>	<i>cagA-EPEAYC</i>	<i>cagT</i>	<i>cagY</i>	<i>cagE</i>	<i>sabA/B</i>	<i>babA2/B</i>	<i>hopQI/II</i>	<i>alpA/B</i>	<i>oipA</i>	<i>iceA1/2</i>
Zak Correlation	-0.283	0.142	0.103	-0.120	-0.035	-0.087	0.094	-0.108	-0.154	-0.059	0.258	-0.055
P-value	0.063	0.357	0.518	0.576	0.870	0.684	0.545	0.616	0.472	0.783	0.224	0.724

Table 6
Analysis of the relationship between gastric diseases, tumor Grade, *vacA* cDNA and *H. pylori* infection variables.

		N	Mean Δ Ct <i>Zak</i>	Standard deviation	Fold Change	p Value
Disease	Gastritis	50	5.0805	2.48714	2.39	0.002
	Gastric Cancer	53	6.3411	1.36349		
Tumor Grade	G1	8	6.0167	1.47822	3.55	0.044
	G3	17	7.8471	1.41712	6.42	0.007
	G2	26	5.1643	1.74512		
	G3	17	7.8471	1.49712		
<i>H. pylori</i> infection	Positive	44	4.9419	2.17727	2.59	0.001
	Negative	59	6.3163	1.80620		
<i>vacA</i> cDNA	<i>s1m1</i>	19	5.6408	2.58277	2.35	0.063
	<i>s1m2</i>	25	4.4108	1.67625		

Basically, with the increasing age of patients, gastric cancer incidence increased, and the *zak* gene expression decreased ($p < 0.05$). Figure 1 shows the nonlinear regression diagrams of *zak* gene expression (Δ Ct) based on patients' age. The result showed a increased Δ Ct with age that indicates to a decrease in *zak* gene expression.

Discussion

The diagnosis of *H. pylori* active infection is considered a risk factor for gastric cancer in long-term (26). Long-term interaction of *H. pylori* with the GAECs and its effects on the genes expression indicates the impact of *H. pylori* chronic active infection to gastrointestinal diseases (27–28).

GAECs react to environmental stimuli, including *H. pylori* infection, by activating or suppressing cellular signaling pathways (29–30). Permanent changes in host cells following long-term stimulation of carcinogens cause suppressing of cell death pathways and tumorigenesis (30). Mitogen-activating kinases are the most common proteins in cells that response to environmental signals (8). Zak protein is one of the proteins that affect carcinogenesis through regulation of cell proliferation (7, 11). Jandhayala et al. showed that ZAK protein is a portion of the cell kinase cascade in response to Shiga toxin and inflammatory genes expression (11). They also showed that suppressing the ZAK protein lead to the prevention of cell apoptosis and inducing carcinogenesis. (11).

The current study results conflicted with Jandhayala et al. study results. The current study found that *zak* gene expression decreased in higher tumor grades of gastric adenocarcinoma, which indicates the antitumor effect of *zak* gene expression. The previous studies show the opposite effects of *H. pylori* CagA and VacA proteins on carcinogenesis and as a fact, *H. pylori* CagA and VacA proteins induces tumorigenesis and apoptosis, respectively. (4). In the current study, *H. pylori* infection had a negative correlation with *zak* gene expression. Our study results showed that infection of *H. pylori vacA s1m2* genotypes compared to *vacA s1m1* genotypes had a weak correlation with *zak* gene expression in GAECs ($p < 0.01$) (Table 6). Therefore, it can be concluded that *H. pylori vacA s1m2* genotypes, unlike other *H. pylori* genotypes, can play a role in apoptosis and inhibition of tumorigenesis by impact on *zak* gene expression.

Yang et al. Showed that ZAK protein acts as a tumor suppressor factor through apoptosis and inhibition of cancer cell growth (7). In a study based on complete sequencing of five pairs of human cancer and natural cells by Cui and et al., the partial sequences of *H. pylori* were found in the early stages of gastric cancer (16). They also showed that *H. pylori* infection causes mutations and deactivation into various genes related to apoptosis, including *zak* (16). Their finding is similar to the results of current study on tumorigenesis of *H. pylori*.

Conclusion

This study showed that *zak* gene expression have a negative correlation with increasing gastric adenocarcinoma tumor grade and *H. pylori* infection.

Declarations

Acknowledgments:

This study results from Mrs. Delniya Khani (MSc Medical Microbiology) and Doctor Manouchehr Ahmadi Hedayati (Ph.D. of Medical Bacteriology) thesis. This study was supported and funded by the Kurdistan University of medical sciences by code number IR.MUK.REC.1397/195. The authors thank Doctor Bahram Nikkhoo (Pathologist) and Doctor Bijan Nouri (Biostatistics) to support sampling.

Compliance with Ethical Standards

Conflict of interest; The authors declare that they have no conflict of interest.

Informed Consent; The institutional review board has approved the study as no published patients' names were involved in the research project.

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Figures

Figure 1

A nonlinear regression plot for the relationship between the age of patients and zak gene expression.