Sirt1 Gene Expression and Gastric Epithelial Cells Tumor Stage in Patients with Helicobacter pylori Infection

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Abstract

Introduction: The World Health Organization has categorized Helicobacter pylori as a carcinogen for gastric cancer, which causes human mortality worldwide. A number of studies have shown that H. pylori affects cell signaling in gastric epithelial cells and changes the expression of some proteins such as proinflammatory cytokines. Bacterial infections may alter sirt1 and sirt2 genes expression in inflammatory tissues and cancer cells. In this study, sirt1 and sirt2 genes expression in gastric cancers was surveyed with reference to H. pylori status. Methods: Stomach biopsies were collected from 50 gastric cancer patients, 25 H. pylori-positive and 25 H. pylori-negative as determined by the urea rapid test. Tumor grade was determined by a pathologist. After total RNA extraction from gastric cancer biopsy samples and cDNA synthesis, sirt1 and sirt2 genes expression levels were determined by Real Time PCR and ΔΔCT methods. Results: There was no statistically significant link between H. pylori infection and sirt1 (P<0.899) and sirt2 (P<0.169) genes expression in gastric epithelial cells. However, pathologic findings showed that there is a statistically significant relationship between sirt1 gene expression and the tumor grade (P<0.024). Discussion: A statistically significant association was found between sirt1 gene expression and tumor grade of gastric cancers that could be due to effects on progression of cancer cells infected with H. pylori.

Keywords: Helicobacter pylori- gastric cancer- sirt1- sirt2- tumor stages

Introduction

H. pylori is a gram-negative bacterium that inhabits the stomachs of more than half of the world population (Salari et al., 2009). Due to the fact that H. pylori is known as an important agent of human gastric cancer, more than a dozen studies have focused on the interaction process between the bacterium and host cells.

It has been demonstrated that H. pylori affects gastric epithelial cells by attachment and injection of some secretory proteins such as cagA (Alzahrani et al., 2014; Chichaklu et al., 2016). After the trigger of the cell signaling from cell membrane phosphokinases, gastric epithelial cells stimulate MAPK/ERK pathway in cell signaling as a response to cagA (Sue et al., 2015). MAPK/ERK pathway is a chain of proteins that is activated in cancer cells (Sue et al., 2015). Cell signaling in gastric epithelial cells is a complicated signaling network. As it has been determined, gene expressions and cell status change after a phosphorylation cascade in cell signaling proteins. NF-KB is a multiunit protein at the end of phosphorylation cascade pathway that as a regulatory protein causes gene expression (Wan et al., 2009). Sirt proteins are known as important factors in tissue inflammation and play an important role in the survival of tumor cells (Vachharajani et al., 2016). Sirt proteins are NAD+-dependent protein deacetylases in mammalian cells that regulate important biological pathways such as inflammation, cell cycle, and tumorigenesis (Vachharajani et al., 2016). Recently, some studies have shown that Sirt1 and Sirt2 proteins are required for the inhibition of apoptosis and inflammatory responses in human cells (Busch et al., 2012; Park et al., 2012). It is clear that sirt inhibitors induce cell death through targeting both sirt1 and sirt2.

Sirt1 and sirt2 genes expression in gastric epithelial cells of gastric cancer patients were surveyed with and without H. pylori infection. For the purpose of complementing this study, relation of sirt1 and sirt2 genes expression and tumor grade of gastric cancer biopsy samples were measured by clinicopathological microscopic observations. This study was done on gastric patients refer to Tohid hospital in Sanandaj city in 2016.
Materials and Methods

Sampling
This case-control study was performed on 25 gastric cancer samples without H. pylori infection as the control group and 25 gastric cancer samples with H. pylori infection as the case group. Biopsy samples were collected from antrum, cardia, body, and fundus area of gastric cancer patients’ stomachs. Gastric cancer biopsy samples were collected from Tohid Hospital in the city of Sanandaj in 2016. Gastric cancers were confirmed by endoscopic and clinicopathological microscopic observations performed by a gastroenterologist and a pathologist, respectively. Two samples were collected from each patient. One for molecular processing and another for urea rapid test. One gastric biopsy sample of the patients was placed in RNA Later for performing molecular tests. Positive urea rapid test samples were considered as positive H. pylori infection.

Real-Time PCR
50 ml/gr from each stomach biopsy were used for total RNA extraction. Biopsy samples were digested in a microtube by Total RNA Extraction kit (Parstous Company, Iran) according to the manufacturer’s instructions. Determination of extracted RNA Integrity was carried out by certain quality and quantitative methods. For quantitative measuring, 3 μl total RNA were mixed with 97 μl diluted water and photobseption was measured at 260 nm using a spectrophotometer machine. Electrophoresis was used on the extracted RNA over 1% agarose gel for qualification testing. The cDNA was synthesized by Easy™ cDNA Synthesis Kit (Parstous Company, Iran). Real-Time PCR was performed for all the samples using specific primers (Table 1) of sirt1 and sirt2 genes, and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) as a reference gene by Corbett machine (Rotor Gene 6000). Table 2 shows the Real-time PCR program for sirt1, sirt2, and GAPDH genes (Zandi et al., 2018).

Determination of Tumor Grade
We collected data of tumor grades for each sample from pathology laboratory of Doctor Bahram Nikkhou in Sanandaj city. For each biopsy sample, a tumor grade microscopic test was performed by a pathologist. Four tumor grades of I, II, III, and IV were determined. The higher the grade, the more malignant the tumor.

Statistical Analyses
Sirt1 and sirt2 genes expression were surveyed in gastric epithelial cells in both groups of patients by relative quantitative Real-Time PCR (Rotor Gene 6000). Sirt1 and sirt2 genes expression in relation to GAPDH as a reference gene in gastric epithelial cells were measured by Ct variations and $2^{-\Delta\Delta C T}$ (Ct test- Ct reference) formula. Veracity of real-time PCR was determined by making dilution from samples and drawing a standard curve. SPSS software 16 and t-test, ANOVA, and Kruskal-Wallis tests were used for data analysis. Average, standard, minimum, and maximum deviations were calculated for each group of data. P-values <0.05 were considered as statistically significant results.

Results

Sampling
All stomach biopsy samples were collected from gastric cancer patients. 15 (%30) samples were collected from Cardia area, 18 (%36) from Antrum area, 6 (%12) from body area, and 4 (%8) samples form Fundus area. The biopsy samples with positive urea rapid test were considered as H. pylori positive infection. Gastric cancer was confirmed by a gastroenterologist’s endoscopic observations as well as clinicopathological microscopic observations. The age range for gastric cancer patients was from 39 to 82. Thirteen and 37 biopsy samples were collected from women and men, respectively. Table 3 shows the respective demographic data.

Relative Quantitative Real-Time PCR
$\Delta C T$ average and standard deviation were calculated for sirt1 and sirt2 genes expression in both the samples of gastric cancer patients with H. pylori infection (case group) and the samples of gastric cancer patients without H. pylori infection (control group). Sirt1 and sirt2 genes expressions in two groups of gastric cancer patients and gastric cancer grades. Results showed that $\Delta C T$ average for sirt1 gene expression in grades I, II, III, and IV of gastric cancer were 6.72, 7.21, 8.23, and 6.59 ± 1.68 respectively (Table 4). Comparison of sirt1 and sirt2 genes expressions in two groups of gastric cancer patients with T-test analysis showed a statistically significant correlation between sirt1 and sirt2 genes expression and H. pylori infection (p<0.889 and p<0.169 respectively).

Clinicopathological Microscopic Test
Forty six biopsy samples had acceptable differentiation for detection of a tumor stage. Twenty (%40) biopsy samples were in grade 1 of tumor stage, 13 (%26) in grade 2, 9 (%18) in grade 3, and 4 (%8) in grade 4 tumor stage. Tumor grade of 4 (%8) biopsy samples were unknown. One-way ANOVA and Kruskal-Wallis tests were used to determine the statistical relationship between sirt1 and sirt2 genes expression in gastric epithelial cells of gastric cancer patients and gastric cancer grades. Results showed that $\Delta C T$ average for sirt1 gene expression in grades I, II, III, and IV of gastric cancer were 6.72, 7.21, 8.23, and

Table 1. Special Primers for sirt1, sirt2 Genes and GAPDH as a Housekeeping Gene

| Gene     | Forward primers          | Revers primers          |
|----------|--------------------------|-------------------------|
| GAPDH    | 5’GCCAGCGGCGACACATC3’    | 5’TGACCGACAGCGCCATAAC3’ |
| Sirt1    | 5’TCCGAACTATACCCGAAACATAGA3’ | 5’CTGTGGAAGGAACCATGACA3’ |
| Sirt2    | 5’GAACAGGAGGACTTGGTGGA3’  | 5’GGGTCACCTCAGAGAAGAT3’ |
8.95 respectively (Table 5); and sirt1 gene expression increases with the increase of gastric cancer grade. These results show that there is a statistically significant correlation between sirt1 gene expression and grade of disease (p<0.024). Δct average for sirt2 gene expression in grades I, II, III, and IV of gastric cancer were 6.04, 6.49, 6.98, and 6.25 respectively; and sirt2 gene expression increases with increase of gastric cancer grade. These results show that there is not a statistically significant correlation between sirt2 gene expression and grade of disease (p<0.364). Kruskal-Wallis test was used to determine the relationship between sirt1 and sirt2 genes expression with tumor differentiation, and there was not a significant statistical association between the two.

### Discussion

According to the World Health Organization statistics, the frequency of new cancer cases in year 2000 was 10.5 million and this number will probably be more than 30 million in 2020 (Karimi et al., 2014). As of now, more than 25 million people worldwide are suffering from cancer and 11 million more develop this disease annually (Karimi et al., 2014). Based on evidence, cancer will increasingly become an important factor in the global burden in the coming decades. After breast and colon cancers, the most common cancer in men and the third most common cancer in women of Iran is the stomach cancer (Mehrabani et al., 2013).

More than a dozen risk factors have been introduced to be involved in increased cancer rates such as High fat, salty diet, foods with high amounts of nitrate, being infected by *H. pylori* and Epstein-Barr Virus, genetic factors, pre-malignant lesions of the stomach, and using tobacco (Zali et al., 2011). Prevalence of *H. pylori* infection and gastric cancer are lower in developed countries in comparison with developing countries (Eshraghian et al., 2014). Prevalence of malignant lesions of the stomach, and using tobacco (Zali et al., 2011). Prevalence of *H. pylori* infection and gastric cancer are lower in developed countries in comparison with developing countries (Eshraghian et al., 2014).

The World Health Organization has classified *H. pylori* as a human carcinogen (Ishag et al., 2015). CagA protein as a *H. pylori* carcinogen-protein results in proliferation of gastric epithelial cells by causing interactions in gastric epithelial cells signaling pathways (Alzahrani et al., 2014). CagA protein activates erk-stat3 signaling pathway that results in inhibition of apoptosis and increase of proliferation in gastric epithelial cells (Alzahrani et al., 2014). Recently, Sirt family proteins have been introduced as key intermediates in the process of inflammation and infection. Some studies have shown that the level of sirt genes expressions is difference in different types of infections (Busch et al., 2012; Park et al., 2012). These studies shows Sirt1 and Sirt2 proteins have conflict results in tumorigenesis. Busch et al., (2012) showed Sirt1 protein is required for the Inhibition of apoptosis and inflammatory responses in human tenocytes. Park et al., (2012) showed Sirt2 is a tumor suppressor protein that connects aging, acetylmelone, cell cycle signaling, and carcinogenesis. We showed sirt1 gene expression increased with progress of tumor stag in gastric epithelial cells of gastric cancer patients. Our results have conflict with result of study of Busch et al. We can find reasons for this contradiction in types of cells and stage of tumorigenesis. Blocking Sirt1 and Sirt2 proteins causes up-regulation of TNF-α and IL-8 genes, that will result in increased inflammation, while up-regulated sirt1 and sirt2 genes cause the decrease of inflammation by deacetylation of NF-KB (Balaiya et al., 2017).

Sirt1 and sirt2 genes expression in gastric epithelial cells of gastric cancer patients with *H. pylori* infection were surveyed. In this study, it was found that sirt1 gene expression has a statistically significant relationship with the stage of gastric cancer. A statically significant association was found between sirt1 and sirt2 genes expression with *H. pylori* infection in the gastric cancer patients. Increase of sirt1 gene expression in advanced stages of stomach cancer pointed to the potential role of Sirt1 protein in the progress of gastric cancer. Sirt1 protein can deactivate p53 as a repressor of tumor by deacetylation (Busch et al., 2012). Some studies have shown a reduction in the level of mRNA and the protein of sirt2 gene in gastric cancer, Melanoma, and Glioma cells (Park et al., 2012). Sirt2 as a cell signaling protein, reduces the process of mitosis through genomic instability and control of cell tumorigenesis activity, and this activity of Sirt2 protein is attributed to deacetylation of p53 as a

### Table 2. Real-Time PCR Program

| Step                | Temperature | Time  | Number of cycles |
|---------------------|-------------|-------|------------------|
| UNG (uracil-N-glycosylase) pre-treatment | 50°C        | 2 min | 1                |
| Initial Denaturation | 95°C        | 10 min| 1                |
| Denaturation         | 94°C        | 15 sec| 45               |
| Annealing            | 60°C        | 30 sec|                  |
| Extension            | 72°C        | 30 sec|                  |

### Table 3. Demographic Data of the Studied Population

| H. pylori Positive Gastric Cancer | Male:Female | Range (Years) | Mean Age (Years) |
|----------------------------------|-------------|---------------|------------------|
| 25                               | 9:16        | 43-78         | 52.22            |

| H. pylori Negative Gastric Cancer | Male:Female | Range (Years) | Mean Age (Years) |
|----------------------------------|-------------|---------------|------------------|
| 25                               | 4:21        | 39-82         | 50.78            |

### Table 4. sirt1 and sirt2 Genes Expression in Gastric Cancer Patients with and without *H. pylori* Infection

| Group | N   | Δct sirt1 | Δct sirt2 |
|-------|-----|----------|----------|
| Case (*H. pylori*) | 25 | 7.3      | 5.96     |
| Control (*H. pylori*) | 25 | 7.35     | 6.59     |

### Table 5. Relationship of Grade of Tumor with Variations of sirt1 and sirt2 Genes Expression in Gastric Epithelial Cells

| Grade of Tumor | N | Δct sirt1 | Δct sirt2 |
|----------------|---|----------|----------|
| I              | 20 | 6.72     | 6.04     |
| II             | 13 | 7.21     | 6.49     |
| III            | 9  | 8.23     | 6.98     |
| IV             | 4  | 8.59     | 6.25     |
subunit of NF-KB (Park et al., 2012). In the present study, sirt2 gene expression was increased in gastric epithelial cells of gastric cancer patients; but this increase of was not statistically significant. On the other hand, tumor stages of biopsy samples of gastric cancer patients were surveyed pathologically. A statistically significant association was found between tumor stages and sirt1 gene expression. Gastric cancer biopsy samples had an increase in sirt1 gene expression that was statistically significant and is compatible with the results of other studies (Noguchi et al., 2014).

In conclusion, these results show that the decrease of sirt1 gene expression coincides with tumor progression. Sirt1 protein has a negative effect on gastric epithelial cell proliferation via an intracellular event. Measuring sirt1 gene expression in gastric epithelial cells can be used as a prognostic marker in detection of progression of stomach cancer; and promotion of sirt1 gene expression may be used as a strategy in inhibition of gastric cancer progression.

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References

Alzahrani S, Lina TT, Gonzalez J, et al (2014). Effect of Helicobacter pylori on gastric epithelial cells. World J Gastroenterol, 20, 12767-80.

Balaiya S, Abu-Amero KK, Kondkar AA, Chalam KV (2017). Sirtuins expression and their role in retinal diseases. Oxid Med Cell Longev, 15, 3187594.

Busch F, Mobasheri A, Shayan P, Stahlmann R, Shakibaei M (2012). Sirt-1 is required for the inhibition of apoptosis and inflammatory responses in human tenocytes. J Biol Chem, 287, 25770-81.

Chichaklu AH, Hedayati MA, Esmailli FS, Ghaderi E, Shiri MHD (2016). Prevalence of H. pylori infection increases HopQ alleles and relation-ship between cagA and vacA S1 With hopQI gene in Helicobacter pylori strains isolated from patients with pep-tic ulcer referred to Towhid hospital in Sanandaj (2014). Biosci Biotech Res Asia, 13, 91-94.

Eshraghian A (2014). Epidemiology of Helicobacter pylori infection among the healthy population in Iran and countries of the eastern mediterranean region: A systematic review of prevalence and risk factors. World J Gastroenterol, 20, 17618-25.

Ishaq S, Nunn L (2015). Helicobacter pylori and gastric cancer: a state of the art review. Gastroenterol Hepatol Bed Bench, 8, 6-14.

Karimi P, Islami F, Anandasabapathy S, Freedman ND, Kamangar F (2014). Gastric cancer: descriptive epidemiology, risk factors, screening, and prevention. Cancer Epidemiol Biomarkers Prev, 23, 700-13.

Mehrabani D, Hosseini SV, Rezaianzadeh A, et al (2013). Prevalence of stomach cancer in Shiraz, Southern Iran. J Res Med Sci, 18, 335-7.

Noguchi A, Kikuchi K, Zheng H, et al (2014). SIRT1 expression is associated with a poor prognosis, whereas DBC1 is associated with favorable outcomes in gastric cancer. Cancer Med, 3, 1553-61.

Park S-H, Zhu Y, Ozden O, et al (2012). SIRT2 is a tumor suppressor that connects aging, acetylene, cell cycle signaling, and carcinogenesis. Transl Cancer Res, 1, 15-21.

Safarzadeh MA, Shirazi MH, Daryani NA (2009). Frequency of Helicobacter pylori vacA genotypes in Iranian patients with gastric and duodenal ulcer. J Infect Public Health, 2, 204-8.

Sue S, Shibata W, Maeda S (2015). Helicobacter pylori-induced signaling pathways contribute to intestinal metaplasia and gastric carcinogenesis. Bio Med Res Int, 12, 737621.

Vachharajani VT, Liu T, Wang X, et al (2016). Sirtuins link inflammation and metabolism. J Immunol Res, 11, 8167273.

Wan F, Lenardo MJ (2009). Specification of DNA binding activity of NF-kB proteins. Cold Spring Harb Perspect Biol, 1, a000067.

Zali H, Rezaei-Tavirani M, Azodi M (2011). Gastric cancer: prevention, risk factors and treatment. Gastroenterol Hepatol Bed Bench, 4, 175-85.

Zandi S, Hedayati MA, Mohammadi E, Sheikhsemaeli F (2018). Helicobacter pylori infection increases sirt2 gene expression in gastric epithelial cells of gastritis patients. Microb Pathog, 20, 120-3.