RESEARCH ARTICLE

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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

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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 referee to Tohid hospital in Sanandaj city in 2016.

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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 photoabsorption 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 *sirtr1* 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 *sirtr1*, *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 $2^{-\Delta\Delta cT}$(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

Δct 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). *Sirt*1 and *sirt2* genes Δct average and standard deviations in the case and control groups were 7.30 ± 1.13, 7.35 ± 1.91 and 5.96 ± 1.50, and 6.59 ±1.68 respectively (Table 4). Comparison of *sirt1* and *sirt2* genes expressions in two groups of gastric cancer patients by 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 Δct 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 *sirtr1*, *sirt2* Genes and GAPDH as a Housekeeping Gene

Gene	Forward primers	Reveres primers
GAPDH	5′GCCAGCCGAGCCACATC3′	5′TGACCAGGCGCCCAATAC3′
Sirt1	5′TCGCAACTATACCCAGAACATAGACA3′	5′CTGTTGCAAAGGAACCATGACA3′
Sirt2	5′GAACAGGAGGACTTGGTGGA3′	5′GGCGTCACCTCAGAGAAGAT3′

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

Patients	Total Number	Male;Female	Range (years)	Mean age (Years)
H. pylori positive gastric cancer	25	9;16	43-78	52.22
H. pylori negative gastric cancer	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

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).

The World Health Organization has classified *H. pylori* as a human carcinogen (Ishaq 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 actives 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, acetylome, 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

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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