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Investigation of Anti-CA II Antibodies in The Sera of *Helicobacter Pylori* Infected Patients

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ABSTRACT

It has been postulated that there may be a correlation between gastric infection caused by *Helicobacter pylori (H. Pylori)* and autoimmune pancreatitis (AIP). An increase in carbonic anhydrase II (CA II) autoantibodies was found in the sera of patients with AIP and it was suggested that measurement of these autoantibodies could be used in the diagnosis of AIP. In this study, the levels of CA II autoantibodies have been determined in the sera of the patients with *H. pylori*-infected gastritis and gastric ulcer. Anti-CA II antibody levels in the sera of these individuals were measured by ELISA method. There were significant differences between the control group and the *H. pylori*-infected gastric ulcer group and the gastritis group. When the *H. pylori*-infected and non-infected gastric ulcer and gastritis groups were compared, it became apparent that there were notable differences. The results indicated that the CA II antibodies in the sera of the *H. pylori*-infected patients were present at a rate of 8.3%. This led to the conclusion that CA II antibodies may not be a reliable marker for *H. pylori* infection. However, the investigation did demonstrate a clear increase in the levels of CA II antibodies for *H. pylori*-infected patients.

Keywords: *Helicobacter pylori*, anti-CA II, autoimmune disease, ELISA.

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Introduction

It has been suggested that *Helicobacter pylori*, a bacterium that has been linked to conditions such as peptic ulcer, gastritis and stomach cancer, may encode carbonic anhydrase II (CA II, EC 4.2.1.1), which could be a potential antibacterial drug target [1]. It is thought that the enzyme is involved in a number of important physiological processes in various tissues, including the transport of CO_2 and ions, renal and male reproductive tract acidification, electrolyte secretion, the formation of gastric acid, bone resorption, ureagenesis, gluconeogenesis, lipogenesis and body fluid generation [2,3]. It is interesting to note that *H. pylori* produces two distinct forms of HpCA, α and β, which exhibit differential localisations within the bacterial cell. α-HpCA, which is highly homologous to CA-II, is anchored to the surface of the bacterium. Consequently, it represents an optimal target for host immunity, as a surface antigen. From a functional perspective, α-HpCA appears to play a role in gastric colonization and acid acclimation in the gastric environment, which could be important for the survival and proliferation of *H. pylori* [4].

Autoimmune diseases are understood to originate from the immune responses of the body to its own antigens [5]. Autoimmune pancreatitis (AIP) is a specific form of pancreatitis that may present with obstructive jaundice, pancreatic masses, lymphoplasmacytic infiltrate and fibrosis, and a marked response to steroids. AIP is estimated to account for 4.6–6% of all chronic pancreatitis cases and is often associated with other autoimmune diseases, particularly Sjögren's syndrome [6]. Inagaki et al. [7] were the first to report the presence of anti-CA II antibodies in the sera of patients with systemic lupus erythematosus (SLE) and Sjögren's syndrome (SjS). Furthermore, it was demonstrated that autoimmune sialoadenitis and immune-mediated pancreatitis could be induced in animal models by immunization with human CA II. Subsequent studies have demonstrated the presence of autoantibodies against CA I and/or CA II in sera from patients with a range of autoimmune disorders, including SjS, SLE, endometriosis, type 1 diabetes, primary biliary cirrhosis, idiopathic chronic pancreatitis, and ulcerative colitis [3]. Antibodies against human CA-II, an enzyme located in the pancreatic ductal epithelium, and lactoferrin are frequently present in the serum of patients affected by AIP, thus suggesting a role for these proteins as autoantigens in the disease [8,9]. In particular, the significance of anti-CA-II autoantibodies in the diagnosis of AIP has been recently corroborated [10]. The theory of microbiological triggering of autoimmunity has been proposed for several decades, based on epidemiological data indicating an increased prevalence of autoimmune diseases following specific infections. A relationship between gastric infection by *H. pylori* and AIP has been reported in the scientific literature. An increase in anti-CA II antibodies has been demonstrated in AIP, and their measurement in the sera of AIP-affected subjects has

been proposed as a diagnostic tool [11]. Furthermore, a striking similarity between the *H. pylori* α-hpCA enzyme and CA II has been demonstrated through *in silico* methods. It has been postulated that α-CA antibodies may exhibit analogous autoimmune effects as CA II autoantibodies [12,13]. In order to confirm the *in silico* results by in vitro methods, the levels of CA II autoantibodies have been determined in the sera of patients with *H. pylori*-infected gastritis and gastric ulcer.

Materials and Methods

Determination of Study Groups

Once the approval had been received from the Karadeniz Technical University Medical Faculty Ethical Committee, Trabzon, Turkey, informed consent was obtained from all subjects (2006/18). Patients with *H. pylori* diagnosed by a gastroenterology consultant and healthy volunteers were recruited. The groups were formed according to the patients' status and the presence of *H. pylori*, as detailed in Table 1. The gender of the patients was not taken into account when the groups were formed.

Table 1. The ages and gender distrubition of the study groups

Groups	Control	*H.pylori* (-) ulcer	*H.pylori* (-) gastritis	*H.pylori* (+) ulcer	*H.pylori* (+) gastritis
Age distribution	19-66	22–65	25-67	25-70	24–68
Sex (male/female)	19/11	13/17	16/14	13/17	10/20

Enzyme-linked immunosorbent assay (ELISA) for serum antibody to CA II

The ELISA method developed by Hosoda et al. [14] was modified and used to determine CA II autoantibody levels of the individuals in the study groups. Measurements were repeated twice for each sample.

Assay protocol

Blood samples were collected from the brachial vein using the venipuncture technique at the time of presentation. Serum specimens were obtained using Vacutainer tubes and subjected to centrifugation at 3000 rpm for a period of 10 minutes. The specimens to be utilized for the measurement of anti-CA II antibody blood concentrations were transferred into Eppendorf tubes and stored at a temperature of -80°C. Human CA II, purified from erythrocytes by electrophoresis, was purchased from Sigma Chemical Co. (St. Louis, MO). The presence of anti-CA II antibodies in serum was determined by ELISA. In brief; the wells in the ELISA plate were coated with 50 µL of CA-II diluted in coating buffer (carbonate buffer 0.05 mM, pH: 9.6) with a concentration of 10 µg/mL and incubated at +4°C for 18 hours.The plate was washed with phosphate buffer (pH 7.0) 3 times at 5 min intervals. After washing, 200 µL of blocking buffer (2% skim milk in phosphate buffer) was added to the wells and incubated on a shaker for 2 hours at room temperature. After blocking, the plate was washed 3 times with phosphate buffer containing 0.05% Tween-20. The wells were incubated with 100 µL of serum diluted with dilution buffer (1:200) for two hours at room temperature. Following the washing step, each well was incubated with 100 µL antibody (peroxidase-conjugated anti-human IgG anti-serum) diluted 1/2000 with dilution buffer containing 1% BSA and kept on shaker for two hour at room temperature. The plate was washed 3 times with phosphate buffer containing 0.05% Tween-20. After washing, the wells were incubated with 100 µL of substrate solution and kept in the dark for 25 minute at room temperature. The reaction was stopped by pipetting 100 µL of 2M H_2SO_4 into all wells. Absorbance was read at 485 nm on an ELISA reader.

All assays were performed in duplicate, and the specific binding of serum antibody to CA II was calculated as follows: the average absorbance of the antigen-coated wells was subtracted from the average absorbance of the control wells (specific binding = A_{coated} - $A_{control}$)

Statistical Analysis

SPSS (IBM SPSS Statistics 17) programme was used for statistical analysis of the data. The conformity of the groups to normal distribution was determined by Kolmogorov-Smirnov test. The statistical difference between the mean values of the groups was tested by One-way ANOVA and Post Hoc Tukey test. Results were expressed as mean and standard deviation (X±SD). $p<0.05$ was considered statistically significant.

Results

The sera of 30 control, 30 H. Pylori negative ulcer, 30 H.pylori negative gastritis, 30 H.Pylori positive ulcer, 30 H.Pylori positive gastritis patients were analysed and the binding of CA II autoantibodies against pure human CA II was determined with ELISA method. Sera with a mean absorbance value > 0.437 were considered to have a positive reaction. This value was calculated by adding 3 SD values (0.225) to the mean absorbance value (X=0.212) of the sera of the control group. (X±S.D) values of the other groups are shown in Table 2. According to the cut off value of the control group, there were 0 positive results in the control group, 2 (6.6%) in the *H.Pylori* (-) ulcer group, 1 (3.3%) in the *H.Pylori* (-) gastritis group, 2 (6.6%) in the *H.Pylori* (+) ulcer group and 3 (10%) in the *H.Pylori* (+) gastritis group. As stated, no positive results were found in the control group (Figure 1). The values showing the significance level of the difference between the groups are given in Table 3. The antibody-positive prevalence rate and the mean absorbance value of *H.Pylori* (+) ulcers (0.307 ± 0.119) and *H. Pylori* (+) gastritis (0.291 ± 0.119) were found to be significantly higher compared with that of control subjects. There was no significant difference in

the prevalence rates and the mean absorbance value between *H. Pylori* (-) ulcers (0.253 ± 0.088) and *H. Pylori* (-) gastritis (0.192 ± 0.083) and control subjects. The antibody-positive prevalence rate and the mean absorbance value of H. pylori-(+) ulcers (0.307 ± 0.119) were found to be significantly higher than those of H. Pylori (-) ulcers (0.253 ± 0.088). The antibody-positive prevalence rate and the mean absorbance value of H. Pylori (+) gastritis (0.291 ± 0.119) were found to be significantly higher than that of H. Pylori (-) gastritis (0.192 ± 0.083). There was no significant difference in the prevalence rates and the mean absorbance value between H. Pylori (+) gastritis (0.291 ± 0.119) and H. Pylori (-) gastritis (0.192 ± 0.083).

Table 2. Serum Anti CA II antibody levels in groups

	Control	*H.pylori* (-) ulcer	*H.pylori* (-) gastritis	*H.pylori* (+) ulcer	*H.pylori* (+) gastritis
Anti CA II antibody	0.212±0.075	0.253±0.088	0.192±0.083	0.307±0.119	0.291±0.119

The data were expressed as mean ± standard deviation (X±SD).

Table 3. p values between the groups (* statistically signficant p<0.05)

Groups	Control	*H.pylori* gastritis	(-) *H.pylori* ulcer	(-) *H.pylori* (+)gastritis	*H.pylori* (+) ulcer	
Control		0.980	0.062	*0.001	*0.00041	P
H.pylori (-) gastritis	0.980	0.104		* 0.00038		P
H.pylori (-) ulcer	0.062			* 0.048		P
H.pylori (+) gastritis	*0.001	* 0.00038				P
H.pylori (+) ulcer	*0.00041		* 0.048			P

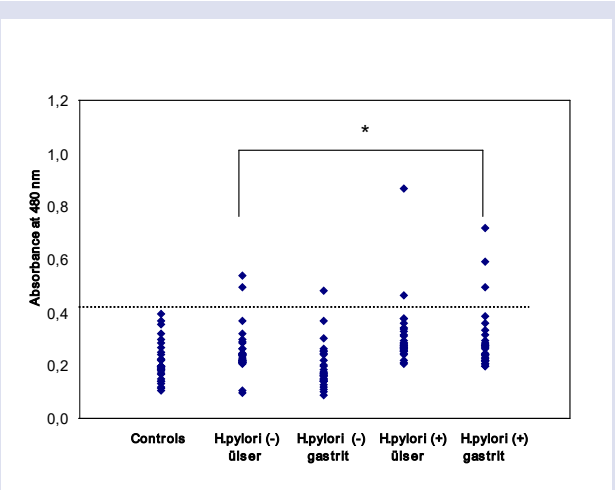

Figure. 1 The presence of anti-CA II antibodies in sera from patients and healthy controls was investigated. The dotted line indicates the mean value plus three standard deviations of healthy control sera (A_{480} = 0.437). A significant difference was observed in the comparison of the mean ± SD value between GD and normal controls ($p < 0.05$).

Discussion

Carbonic anhydrases are ubiquitous metalloenzymes present in prokaryotes and eukaryotes (14). CA II is widespread enzyme, present in mammals tissues. It catalyzes the irreversible hydration of CO_2 provides bicarbonate, a substrate for many reactions and in acid-base equilibrium and plays important roles in regulation of cardiovascular tonus and control of ion exchange between cell compartments [15]. Autoantibodies may be developed response to injured or antigenically altered tissues. In 1991, Inagaki et al. [7] first investigated anti CA II antibodies in the sera of patients of systemic lupus erythematosus and Sjögren's syndrome. It couldn't be explained that as a sytoplasmic enzyme how CA II antibodies causes cellular damage that seen in autoimmune diseases. But in the recent years the investigation of anti CA II antbodies in the sera of patients with autoimmune pancreatitis (AIP) gave rise to tought use of the positivity of anti CAII antibodies as a criteria in the diagnosis of this disease. Guarneri et al. [11] mentioned that subjects infected by Helicobacter pylori and have genetically bent have had AIP and AIP-anti CAII and based on the occurrence of AIP in some genetically predisposed individuals and the association of AIP-anti CA II, they determined a large amount of homology between the H. pylori α-hpCA enzyme and CA II in a fully computerized (in silico method). It was reported that α-hpCA antibodies may exhibit autoimmune effects similar to anti CA II.

In this study, *in vitro* determination in the sera of *H. pylori* infected individuals was aimed according to the hypotesis of Guarneri et al. suggested the data obtained from *in silico* method. For this purpose, five study group of thirty subjects each of those was evaluated by a gastroeneterologist were formed.The ELISA method developed by Hosoda et al. [16] was used with minor modifications to investigate the anti-CA II antibodies in the sera of the individuals in the study groups. The results and the standart deviations obtained from ELISA are shown in table 2. The cut off point that was used for evaluating the positivity of the study groups was taken as the avarage of the control group sum with 3SD ($X \pm 3SD$= 0.437) , because if it was evaluated among 3SD there would be no positive result in the control group and this enhanced the reliability of the method. The results of the groups are given together in the same graph (Fig 1.). It is seen that there are 3 positive in *H.pylori* (+) gastritis group (10 %), 2 in *H.pylori* (+) ulcer group (6,6 %), 1 in *H.pylori* (-

) gastritis group (3,3 %), 2 in *H.pylori* (-) ulcer (6,6 %) and in the sum 8 positive results were obtained (5,3 %) . Here, anti CA II antibodies were detected in the sera of only 5 (8,3%) of 60 *H.pylori* infected subjects with the ELISA method. It was concluded that altough there was no positive value in the control group,as a good indicator of high specificity, this number meant a low sensivity, so determination of anti-CA II antibodies in the serum with ELISA could not be a marker for *H.pylori* infection. One positive value in *H.pylori* (-) gastritis group and in *H.pylori* (-) ulcer could be arise from some subjects of these groups might have a treated *H.pylori.* as a result of advanced statistical anlaysis between control group and *H.pylori* (+) gastritis and ulcer groups significant difference was found. (respectively, p=0,001 ve p=0,00041). We do not have chance to compare our results, because of the lack of study in this issue. But when compared with the control group subjects with *H.pylori* (+) ulcer had increased anti-CA II antibodies levels rather than subjects with *H. pylori* (+) gastritis and this difference has been arised from the fact that worse damage and localization of ulcer. Likewise, differences between the *H.pylori*(+) and *H.pylori*(-) gastritis groups and *H.pylori*(+) and *H.pylori*(-) ulcer groups were statistical significant (respectively, p=0,00038 and p=0,048). The results were in concordance with our expectations. Increased anti-CA II antibody levels were found in the sera of *H.pylori* infected subjects. But, here increased CA II might not be the real CA, it could also be the hpCA that belongs to *H.pylori*. Because, the role of anti-CA II antibodies in the pathology of autoimmun disease is not clear. Infections are important enviromental factors that triggers autoimmunity and it was reported that *H. pylori* infection induces AIP, an autimmun disease. It is known that, according as necrosis caused by viruses and microbial agents, antibodies can develop with mechanisms such as molecular mimic against spesific enzymes, cross-links, activation and adjuvant effect of autoreactiveT-cells. Anti-CA II antibodies could be developed in *H.pylori* infected individuals with similar mechanisims. It was demonsrated that human CA II shows antigenic character by inducing humoral and cellular immune reactions with studies performed with various rat species those were constituted autoimmun diseaes. It was seen that genetic factors were also determinative in forming of autoantibodies. F.Guarneri et al found significant homology between CA II and shared segments on DRB1*0405, one of binding motifs of hpCA to HLA and suggested that this was a risk factor for AIP. These findings supported the idea of the association between AIP and HLA DRB1*0405 genotype, present in the shared segments with human and bacteria. Further studies on this genotype will be usefull for identification of autoimmune diseases. On the side, the contribution of *H. pylori* positivity and HLA types to the presence of anti-CA II antibodies in the positive results shoud not be undervalued. Carbonic anhydrase has two isoenzymes in *H.pylori*, including alfa and beta and alfa hpCA has considerable homology with human CA. Alfa hpCA exists on the outer surface of bacterium and this makes easy the interaction of host immunity and antibody and the organism can facilely produces antibodies. Because of the similarity between alfa hpCA and human CA II, alfa hpCA antibodies could be cross-linked to CA II, coated to ELISA plate causing increased anti CA II antibodies.

Conclusions and Recommendation

In this study, the hypothesis proposed by Guarneri et al [11], based on the results obtained by in silico methods, was tested in vitro in the sera of H. pylori infected individuals. There were 8 positive values, 3 (10%) in the H. pylori (+) gastritis group, 2 (6.6%) in the H. pylori (+) ulcer group, 1 (3.3%) in the H. pylori (-) gastritis group and 2 (6.6%) in the H. pylori (-) ulcer group, with a cut-off value of <0.437 in the control group. Statistical analysis revealed a significant difference between the control group and the H. pylori (+) ulcer and H. pylori (+) gastritis groups (p=0.00041 and p=0.001 respectively), between the H. pylori (+) ulcer and H. pylori (-) ulcer groups (p=0.048) and between the H. pylori (+) gastritis and H. pylori (-) gastritis groups (p=0.00038). After evaluating the results obtained, it is recommended that the following additional study be carried out to obtain more conclusive information on this subject*:* *H.pylori* alpha-hpCA enzyme can be purified, ELISA plates can be coated with this enzyme and anti-CA II can be measured and compared with measurements of CA II coated plates. It can be determined whether the antibody is anti alpha-hpCA or anti CA II.

Conflicts of interest

There are no conflicts of interest in this work.

Ethical Approval Statement

This study was approved by the Karadeniz Technical University Medical Faculty Ethical Committee (Protocol no: 2006/18) and informed consent was obtained from all subjects.

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