Detection of autoantibodies against carbonic anhydrase I and II in the plasma of patients with gastric cancer

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Abstract
Cancer is the second leading cause of death and gastric cancer is the fourth most common cancer type worldwide. Investigation of autoantibodies in cancer patients has been a popular research area in recent years. The aim of the current study was to investigate carbonic anhydrase I and II (CA I and II) autoantibodies in the plasma of subjects with gastric cancer based on the information and considerations of autoimmune relation of gastric cancer. Anti-CA I and II antibody levels were investigated by ELISA in plasma samples of fifty two patients with gastric cancer and thirty five healthy peers. Anti-CA I and II antibody titers of the gastric cancer group were significantly higher compared with the control group (p = 0.004, p = 0.0001, respectively). Plasma anti-CA I levels of the metastatic group were lower than the non-metastatic group and this difference was found statistically significant (p < 0.05), but there was no statistical difference between plasma anti-CA II levels of the groups. CA I and II autoantibody titers in patients with gastric cancer were found higher compared to healthy subjects and the results suggest that these autoantibodies may be involved in the pathogenesis of gastric cancer.

Key words: autoantibody, carbonic anhydrase, gastric cancer, Helicobacter pylori.

Introduction
Malignant diseases progress with stimulation of autoimmunity that is characterized by formation of antibodies against their own antigens, and autoantibodies are detected in the sera of patients with solid tumors [1, 2]. These autoantibodies are evaluated as early biomarkers of some types of cancer [3-5]. Cancer is the second leading cause of death and gastric cancer (GC) is the fourth most common cancer type worldwide [6]. Estimated new events and deaths from this disease in the US in 2015 were 24,590 and 10,720, respectively [7]. Approximately, 80% of gastric adenocarcinomas are associated with Helicobacter pylori infection and occur secondary to associated gastritis [8]. Autoantibodies developed against antigens of H. pylori and self-antigens of the organism are detected in the sera of subjects with GC [9]. Carbonic anhydrase (CA) is a metalloenzyme, which catalyzes the reversible hydration of carbon dioxide to bicarbonate. CA functions in many physiological and pathological processes, such as transport of carbon dioxide, pH regulation, ion transport, formation of stomach acidity, bone resorption, calcification, and tumorigenesis. Thus far 16 isoenzymes that differ from each other in tissue distribution, cell localization, catalytic activity and resistance to inhibitors, are described [10, 11]. Most of these isoenzymes are expressed in the gastrointestinal tract [12]. In recent years CA I and II autoantibodies have been demonstrated in some autoimmune diseases and carcinomas, but mechanisms underlying this immune response have not been explained yet [13, 14].
The aim of our study was to evaluate CA I and II autoantibodies in the subjects with gastric cancer by the ELISA
method and provide a novel perspective to autoimmune basis of gastric cancer.

**Material and methods**

**Study group**

Elucidated approval was obtained from all patients and controls. Approval for the study was given by the local ethics committee. Fifty two newly diagnosed patients with GC (24 metastatic, 28 non-metastatic) as the study group and thirty five healthy peers as the control group were admitted to this study. There were 30 men and 22 women with a median age of 58 (range: 45-70) years in the study group, while there were 16 men and 19 women with a median age of 56 (range: 40-72) years in the control group. Patients were selected from individuals who applied to the Medical Oncology Clinic and were referred from other practitioners. Patients who had renal, coronary and liver failure, chronic inflammatory diseases, anemia, received chemotherapy, oral contraceptives and anticoagulants were excluded from the study. Patients were staged according to the seventh edition of the American Joint Committee on Cancer Manual [15]. Staging was assessed according to this classification. Out of 52 patients with gastric adenocarcinoma, 13.5% (7 patients) had stage II disease, 40.4% (21 patients) had stage III disease and 46.2% (24 patients) had stage IV disease. All patients had the same characteristic cancer cell type as an adenocarcinoma.

Five milliliters blood sample for each individual was obtained in vacutainer tubes with K₃ EDTA. Tubes were centrifuged at 1800 g for 10 minutes. Plasma samples were stored at –80°C until measurements.

**Determination of plasma autoantibody to CA I and II**

Plasma CA I and II autoantibodies were determined by ELISA according to the previously described method [16]. Each sample was assayed in duplicate and the specific binding of plasma antibody to CA I or CA II was calculated as follows:

\[ \text{Specific binding} = \text{OD}_{\text{coated}} - \text{OD}_{\text{control}} \]

**Statistical analysis**

Statistical analysis was applied using a statistical package for the social sciences (Version 13.0, NY, USA) and MedCalc (Version 12.3, Mariakerke, Belgium) statistical software. Suitability for normal distribution was determined by Kolmogorov-Smirnov test. The differences between all groups were analyzed using Student’s t-test for data with normal distribution. The receiver operating characteristic (ROC) curves were used to detect the discriminatory dominance of CA I and II autoantibodies for identification of GC. Sensitivity, specificity, negative predictive values (NPV) and positive predictive values (PPV) were determined with regard to ROC graphs for autoantibodies of CA I and II. \( p < 0.05 \) was regarded as significant.

**Results**

Fifty two GC patients and thirty five healthy subjects were included in our study and 24 of cases were metastatic and the rest were non-metastatic subjects. There was no significant difference in terms of the median age between study and control groups. Levels of CA I and II autoantibodies in patients with GC and control subjects are shown in Figs. 1 and 2, respectively.

The mean absorbance value of CA I autoantibody for the healthy subjects was 0.236 ±0.082 and the absorbance was higher than 0.479, the mean absorbance + 3SD of healthy individuals, were identified as positive. Positive results were obtained in 5 (all of non-metastatic) out of 52 subjects with GC (Fig. 1). The mean absorbance value of the GC group (0.299 ±0.117) was detected to be markedly

![Fig. 1. Anti-CA I antibodies in plasma from patients with GC and healthy controls. The dotted line indicates the mean value + 3SD of healthy control plasma (A<sub>μμ</sub> = 0.479)](image1)

![Fig. 2. Anti-CA II antibodies in plasma from patients with GC and healthy controls. The dotted line indicates the mean value +3 SD of healthy control plasma (A<sub>μμ</sub> = 0.291)](image2)
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Table 1. Anti-CA I and II antibody levels (ABSU)

|                   | Groups         | n  | Patient       | Control       | Metastatic    | Non-metastatic |
|-------------------|----------------|----|---------------|---------------|---------------|---------------|
|                   |                |    | 52            | 35            | 24            | 28            |
| Anti-CA I         | 0.299 ±0.117a  |    | 0.236 ±0.082  | 0.260 ±0.085b | 0.332 ±0.131  |
| Anti-CA II        | 0.194 ±0.133b  |    | 0.087 ±0.069  | 0.196 ±0.120  | 0.192 ±0.159  |

Data are mean values ± SD; *p = 0.004 compared with control, *p = 0.0001 compared with control, *p < 0.05 compared with non-metastatic

Table 2. ROC curve analysis of a CA I and II autoantibodies values and their sensitivity, specificity, PPV and NPV

|                   | Cut-off Point | Sensitivity (%) (95% CI) | Specificity (%) (95% CI) | PPV (%) (95% CI) | NPV (%) (95% CI) |
|-------------------|---------------|--------------------------|--------------------------|------------------|------------------|
| Anti-CA I         | > 0.250       | 61.5 (47.0-74.7)         | 68.6 (50.7-83.1)         | 74.4 (58.8-86.5) | 54.5 (38.8-69.6) |
| Anti-CA II        | > 0.100       | 78.4 (64.7-88.7)         | 73.5 (55.6-87.1)         | 81.6 (67.8-91.3) | 69.4 (51.9-83.7) |

ROC – receiver operator characteristic; PPV – positive predictive value; NPV – negative predictive value

higher (p = 0.004) compared with the healthy subjects (Table 1). The mean absorbance value of CA II autoantibody for the healthy subjects was 0.087±0.069 and the absorbance was higher than 0.291, the mean absorbance +3SD of healthy individuals, were identified as positive. Positive results were obtained in 11 (4 metastatic, 7 non-metastatic) out of 52 subjects with GC (Fig. 2). The mean absorbance value of the GC group (0.194 ±0.133) was detected to be markedly higher (p = 0.0001) than of the healthy subjects (Table 1).

Statistical analysis was performed by dividing the patients into two groups as metastatic and non-metastatic. Plasma anti-CA I levels of the metastatic group were lower than the non-metastatic group and this difference was found statistically significant (p < 0.05), but there was no statistically difference between plasma anti-CA II levels of the groups (Table 1).

Plasma CA I and II autoantibody levels were also evaluated using ROC curve analysis. Cut-off points, sensitivity, specificity, PPV and NPV for the parameters were demonstrated in Table 2 and Figs. 3 and 4.
Discussion

Gastric cancer is the fourth most common cancer type and the second cause of cancer-related death. Late diagnosis is one of the reasons of its high mortality. In recent years, there have been therefore many research studies about identification of a new biochemical diagnostic marker for early detection of GC [17, 18]. Autoantibodies in the blood sample of patients have been proposed as diagnostic biomarkers for early-stage diagnosis of cancers, as an increase in serum levels of certain autoantibodies has been shown to precede the development of disease symptoms and correlate with cancer incidence for many types of cancer [19]. Using serum antibodies as markers for cancer has some advantages. Primarily, cancer related autoantibodies exist in the bloodstream much earlier than serum antigens. Secondly, autoantibodies can have higher levels in the bloodstream compared to antigens [4].

The present study is the first report which shows an increased immune response to both CA I and II in GC patients. We found CA I and II autoantibody prevalence in patients with GC as 9.6% and 21.2%, respectively (Figs. 1 and 2). In this study, an anti-CA I antibody cut-off level of 0.250 ABSU was related to NPV of 54.5% and PPV of 74.4%, with 61.5% sensitivity and 68.6% specificity and an anti-CA II antibody cut-off level of 0.100 ABSU was related to NPV of 69.4% and PPV of 81.6%, with 78.4% sensitivity and 73.5% specificity according to ROC curves. To date, there has been no gastric cancer specific biomarker, although autoimmunity against many autoantigens, such as p53, NY-ESO-1, mucin-1 (MUC1), c-myc, survivin, koc, p62, astrocyte elevated gene-1 protein (AEG-1), matrix metalloproteinase-7 (MMP-7), 70 kilodalton heat shock proteins (Hsp70), carcinoembryonic antigen (CEA) and histone H2B are reported in GC. The sensitivity and specificity of these autoantibody markers range from 0% to 75% and from 71.7% to 100%, respectively [18]. When viewed from this aspect, sensitivity and specificity of CA I and II autoantibodies were similar with previous autoantibody research in GC.

Carbonic anhydrase isoenzymes are virtually ubiquitous in living systems, have various functions in most normal mammalian and bacterial cells. CA I and II are the most widely distributed members of the CA family, being present almost in all tissues [20, 21]. Also, CA I and II have been demonstrated to be associated with gastrointestinal neoplasms and CA II has been mentioned as a new biomarker for gastrointestinal stromal tumors [22]. Increased autoimmunity against these isoenzymes has been reported in cancer and autoimmune diseases, but no mechanisms have been identified [13]. It has also been suggested that genetic, hormonal, and environmental influences may play a part in triggering autoimmunity [19]. The higher levels of autoantibodies in cancer patients can be explained by overexpression, aberrant expression, mutation, changes in protein half-lives, misfolding, aberrant degradation or abnormal posttranslational modification of the proteins [18, 23]. The increase in these autoantibodies may be a secondary epiphenomenon to proliferation of cancer cells. Iuchi et al. demonstrated that SOD-knock out mouse developed CA II autoantibodies as a result of increased oxidative stress [24]. 4-hydroxy-2-nonenal (HNE), one of the end products of lipid peroxidation, modifies proteins and alters antigenic properties of them. Uchida et al. reported that CA II is a target for HNE in their study related with erythrocytes [25]. Increased CA autoantibody titers in the plasma of GC patients may be a result of increased oxidative damage in cancer tissue. Moreover, antibodies against to H. pylori antigens are determined in most of subjects with GC [26]. Helicobacter pylori is a gram negative, microaerophilic bacteria. This bacteria is known as the etiological agent of chronic active gastritis and as the factor responsible for most of the peptic ulcer cases and a co-factor for GC and MALT-lymphoma [27, 28]. Helicobacter pylori has α- and β-carbonic anhydrase enzymes. These enzymes provide acid acclimation of the pathogen in the gastric tract. Helicobacter pylori α-class CA (bpaCA) has been sequenced from individuals with varied gastric mucosal lesions, such as gastritis, ulcer and GC [10]. An in vitro study demonstrated that antibodies against CA of this bacteria might have formed an autoimmune response to human CA as a result of molecular mimicry [29, 30]. In a study from our laboratory, anti-CA II titers of H. pylori positive subjects were found higher than H. pylori negative subjects and control groups (unpublished data). Most of the CA isoenzymes including CA I and II are found in the gastrointestinal system cells. CA IX and XII are related to carcinogenesis and have an increased expression in malignant tumor cells [12]. Immune response to CA I and II in GC subjects may be a cross-reactivity resulting from other CA isozymes that mimic these ones.

There are many methods, such as ELISA, western blot, protein microarrays, agglutination assays, immunoblotting, flow cytometry, immunoprecipitation and immunofluorescence for determination of autoantibodies in the sera of cancer patients. ELISA is frequently used for detection of autoantibodies in blood samples due to its cheapness, easiness and quickness as compared to the other assay [18, 31]. Besides, it has been mostly used for evaluation of CA I and II autoantibodies in different pathological conditions in previous reports [11, 14, 16, 32]. We have therefore preferred to determine autoantibody levels using ELISA in this study.

Plasma anti-CA I levels of the non-metastatic group were higher than the metastatic group and this difference was statistically significant. The finding that in non-metastatic patients elevated the immune response to CA I may have prevented tendency to metastasis. This may be related to the ability of cancer cells to escape from the immune system. For this reason antibody formation in the meta-
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static subjects may be less than in the non-metastatic ones. The major limitation of the current study may be a relatively small number of patients. Further larger-scale prospective and molecular studies are required for a fully understood autoimmunity mechanism of CA I and II in GC.

In the present study, CA I and II autoantibodies are detected in GC subjects, but the pathogenic role of these antibodies remain uncertain. It shows the need for further trials to evaluate the significance of CA autoantibody production in GC subjects.

Ethical Approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

The authors declare no conflict of interest.

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