The relationship between neuroendocrine tumor markers and diabetes: Chromogranins A and B

Doctoral thesis

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Budapest
2020

List of abbreviations

AIG	Autoimmune gastritis
CgA	Chromogranin A
CgB	Chromogranin B
ECL	Enterochromaffin-like
HbA_{1C}	Glycated hemoglobin
LADA	Latent autoimmune diabetes in adults
T1DM	Type 1 diabetes mellitus
T2DM	Type 1 diabetes mellitus

1. Introduction

Chromogranins are family members of the granin glycoproteins, and are expressed by neurons, endocrine and neuroendocrine cells throughout the body. Their original function is the selective protein sorting of various biologically active molecules within secretory granules, but new functions related to their cleavage products have emerged during their evolution. Some of the chromogranin cleavage products are involved in metabolic diseases and psychiatric disorders, while others have antimicrobial activity.

The relationship between chromogranins, carbohydrate metabolism and diabetes mellitus is an ongoing research area: both chromogranin A (CgA) and its cleavage product, and chromogranin B (CgB) was reported to have a significant role in carbohydrate metabolism and diabetes.

A notable connection has been inferred through the observation that type 1 diabetes mellitus (T1DM) is not at all or rarely developed in CgA gene-knockout, non-obese diabetic model mice compared to non-knockout, non-obese diabetic mice. Pancreastatin, one of the CgA cleavage products, suppresses insulin signaling and inhibits insulin release, and it has an elevated serum level in type 2 and gestational diabetes patients. WE-14 and some other small CgA-fragments function as autoantigens for the diabetogenic, pancreatic-cell-destroying $CD4^+$ and $CD8^+$ T-cell populations in both non-obese diabetic mice and patients with type 1 diabetes.

There is some knowledge about the relationship between CgA and the carbohydrate metabolism, while very little is known about CgB. CgB contributes to the physiological secretion of insulin in pancreatic beta cells, and probably takes part in the signal transduction of insulin secretion.

In the present study, serum CgA and CgB level of type 1 and type 2 diabetes patients, and healthy control subjects were investigated along with their relationships to other clinical parameters.

2. Objectives

Aims of the study were:

1) Within the type 1 diabetes patients
   a) To determine the serum CgA level.
   b) To investigate whether serum CgA level is associated with other parameters tested routinely in diabetes care such as glycated hemoglobin (HbA_{1C}), complete blood count or cholesterol levels.
   c) To investigate whether there is a connection between serum CgA level and the various comorbidities associated with diabetes.
   d) To observe if serum CgA level changes with the duration of type 1 diabetes.
2) Within the type 2 diabetes patients
   a) To determine the serum CgA level.
   b) To investigate whether serum CgA level is associated with various laboratory or anamnestic data.
3) Within voluntary, healthy control subjects and type 1 and type 2 diabetes patients
   a) To measure and determine the serum CgB level.
   b) To investigate whether serum CgB level is associated with the measured laboratory results or collected anamnestic data.
   c) To compare CgB level of diabetes patients with those of healthy controls.
   d) To examine the connection between CgA and CgB.

3. Methods

3.1. Patients

A total of 355 study participants with Caucasian ancestry, who attended the Metabolic Outpatient Clinic of the Department of Internal Medicine and Hematology (formerly 2nd Department of Internal Medicine), Semmelweis University were enrolled in the study. The study population consisted of 161 type 1 and 100 type 2 diabetes patients, and 94 voluntary control subjects. Enrollment of T1DM and T2DM patients occurred between 2010-2019 and 2017-2019, respectively. Written informed consent was collected from all study subjects. The study was approved by the Regional and Institutional Committee of Science and Research Ethics, Semmelweis University.

Exclusion criteria included age under 18 years, any kidney diseases, known tumors, mental disorders, ulcerative colitis, Crohn's disease, systemic rheumatoid arthritis or any conditions changing the serum concentrations of CgA such as the use of acid reducer medications. CgB is not affected by acid reducer medications based on data available from the literature, therefore no study subject was excluded from that part of the analysis.

3.2. Study design

In T1DM, an observational cohort study was carried out, where cohorts were defined by the serum CgA level. A subpopulation of patients (n = 34), who had elevated CgA or any other condition that indicated the procedure, agreed to undergo gastroscopy, and 11 of these patients had gastroscopy prior this study, which allowed the retrospective analysis of histopathological changes through frozen biopsy samples. Serum CgA in T2DM, and serum CgB level of control subjects and diabetes patients were determined via cross sectional observational studies.

3.3 Clinical data and measurements

Anamnestic data and body mass index (BMI) were collected; blood samples were drawn after an 8 h fasting period. Complete blood count, glycated hemoglobin (HbA_{1C}), total-, low- and high-density lipoprotein cholesterols, triglycerides, creatinine, high sensitivity C reactive protein and thyroid-stimulating hormone were measured. Serum

gastrin and CgA levels were measured at the Central Endocrine and Genetic Laboratory, Semmelweis University using radioimmunoassay kits, while serum CgB levels were measured with the Human Chromogranin B (CHGB) enzyme-linked immunosorbent assay kit (dilution 10:1, abx151068, Abbexa Ltd., Cambridge, UK) at the Metabolic Laboratory of the Department of Internal Medicine and Hematology, Semmelweis University. Intensive conservative insulin treatment for type 2 diabetes patients was defined as a multicomponent regimen including basal and prandial insulins.

3.4 Gastroscopy and chromogranin A-specific immunohistochemical staining

Gastroscopy was carried out at the Gastroenterology Clinic of the Department of Internal Medicine and Hematology, Semmelweis University. Biopsy samples for routine histological examinations and CgA-specific immunohistochemical staining were collected from the fundus, oral and aboral parts of the antrum, and pylorus.

Histopathological classification of the CgA-positive, enterochromaffin-like (ECL) cell hyperplasia were described as 1.) cells are located randomly and individually in the case of diffuse ECL hyperplasia, 2.) cell clusters form a chain of five or more cells in linear ECL hyperplasia and 3.) cells are orderly arranged, forming knots between 100 and 150 μm in size in micronodular ECL hyperplasia.

3.5 Statistical analysis

Statistical analyses were performed with R for Windows version 4.0.1. Two sample Welch-test, Bayesian methods and permutation-based paired t-tests, Fisher's exact test, Pearson's correlation, Spearman's rank correlation and linear and logistic regression models, receiver operating characteristic (ROC) analysis, propensity score matching and random intercept linear mixed effect models were used. Bayesian p-values were determined based on the density at the Maximum A Posteriori distribution. $P < 0.05$ was considered as statistically significant; p-values were corrected with the false discovery rate method for the multiple comparisons problem. Results were expressed as mean ± standard deviation for continuous data, and as the number of observation (percentage) for count data.

4. Results

4.1. Chromogranin A in type 1 diabetes mellitus

4.1.1 Baseline measurements

Patients were divided into two cohorts based on their baseline serum CgA levels. Study participants having levels within the normal range (19.4 – 98.1 ng/mL) were assigned to the *Normal CgA* group (n = 132 (82%); 49.80 ± 19.51 ng/mL), whereas patients with levels higher than the normal range were assigned to the *High CgA* group (n = 29 (18%); 287.89 ± 244.72 ng/mL).

HbA_{1C} levels in the *High CgA* group were significantly higher (9.68 ± 2.00 %), compared with those in the *Normal CgA* group (8.24 ± 1.93 %; p = 0.0105). A significant positive correlation was found between CgA and HbA_{1C} (Pearson's R: +0.33; explanatory power of the model: 10.17%; p < 0.0001). Based on the HbA_{1C} values, 69.7% of the patients had poor glycemic control ($HbA_{1C} \geq 7.0\%$) in the *Normal CgA* group, whereas this figure rose to 96.6% in the *High CgA* group (p = 0.0291).

4.1.2 Changes in serum chromogranin A levels with the duration of type 1 diabetes

To determine whether the CgA level changes with respect to the duration of type 1 diabetes, we chose two prospective approaches. First, the patients were recalled for a follow-up measurement. We were able to reach 96 patients (call-back rate 59.6%, mean duration between measurements 4.67 ± 2.28 years). CgA levels elevated in the *Normal CgA* group (n = 79, 47.85 ± 19.38 ng/mL vs. 53.88 ± 27.90 ng/mL, p = 0.0191), whereas no change was observed in the *High CgA* group (n = 17, p = 0.2202). The CgA level of five (6.3%) *Normal CgA* patients were above the normal range at the follow-up. In the case of all patients, like in the *Normal CgA* group, a significant increase was observed (n = 96, 104.13 ± 179.13 ng/mL vs. 126.88 ± 302.44 ng/mL, p = 0.0495).

Second, a random intercept linear mixed effect model was constructed. Based on a total of 376 CgA measurements an annual 0.40–1.82% increase in the serum CgA concentrations are be expected (p = 0.0410).

4.1.3 Gastroscopy results

Gastroscopy was carried out in 15 patients from *Normal CgA* and 19 from *High CgA* groups. ECL hyperplasia positivity was more frequent in the *High CgA* patients (odds

ratio (OR): 5.74, p = 0.0087). The distribution of hyperplasia types was significantly different (p = 0.0087): diffuse ECL hyperplasia occurred in both groups, but more advanced forms of hyperplasia were found only in the patients of the *High CgA* group. Histologically-confirmed autoimmune gastritis (AIG) was significantly more frequent in the *High CgA* group (OR: ∞ due to division by zero, p = 0.0087).

4.1.4 Progression of ECL hyperplasia in patients with continuously high serum CgA levels

The examination of archive biopsy samples could be carried out in 11 of the 19 patients in the *High CgA* group (time between sample collections: 3.94 ± 1.96 years). A significantly (p = 0.0192) higher number of more advanced hyperplasia stages were observed at the recent gastroscopic examination compared with those at the archive samples. There was no change in 4 patients at all, and a progression was observed in 7 patients.

The serum CgA levels of patients with and without progression were compared with a one-sided paired t-test, assuming an increase in CgA levels. Serum CgA levels were significantly higher (p = 0.0316) in patients with ECL hyperplasia progression, whereas they did not differ statistically in patients without histological progressions (p = 0.3752).

4.1.5 Case report

A case presented here is a good example of how the regular measurement of CgA level allowed the early detection of a neuroendocrine tumor in T1DM patients. First serum CgA measurement of this male patient occurred in 2006. CgA level was slowly rising, and CgA level was already above the normal range in 2015. In late 2019, patient's serum CgA level had elevated to a large extent and the patient started to complain about mild diarrhea and epigastric discomfort, but stool microbiology test, gastroscopy and colonoscopy had been all negative. Computed tomography (CT) scan of the abdomen was initiated, which showed a hypervascularized area in the mesentery, caudally from the duodenum. This deviation was not visible on a previous abdominal CT scan initiated because of increasing CgA levels in 2015. Fine-needle aspiration confirmed a CgA- and synaptophysin positive, grade I neuroendocrine tumor with low Ki-67 proliferation rate.

The tumor was surgically removed, and histological findings strengthened the biopsy results. One month after tumor removal, serum CgA returned almost into normal range.

4.2 Chromogranin A in type 2 diabetes mellitus

T2DM patients were divided into two cohorts based on their baseline serum CgA level. Study participants having CgA level within the normal range (19.4 – 98.1 ng/mL) were assigned to the *Normal CgA* group (n = 80 (93%), 50.43 ± 21.73 ng/mL), whereas patients with levels higher than the normal range were assigned to the *High CgA* group (n = 6 (7%); 129.33 ± 41.07 ng/mL). No differences were found between the study groups neither in laboratory parameters, nor in anamnestic data.

4.3 Investigation of serum chromogranin B levels

4.3.1 Chromogranin B level of healthy control subjects

A total of 94 healthy control subjects were included in the study. The normal range of CgB has not yet been precisely determined, in the current study the median of CgB was 97.00 ng/mL and the interquartile range was 68.13 – 131.65 ng/mL. Age, sex, estimated glomerular filtration rate, body mass index, antacid therapy and/or known comorbidities did not affect serum CgB levels.

4.3.2 Chromogranin B level of type 1 and type 2 diabetes patients

CgB level was not affected by any other parameters (including HbA_{1C} and duration of diabetes as well) neither in T1DM, nor in T2DM patients. T1DM patients had significantly lower serum CgB level than the corresponding age- and sex matched control subjects (107.38 ± 59.77 ng/mL vs. 89.39 ± 34.23 ng/mL, p = 0.0241). No correlation was found between serum CgA and CgB levels (p = 0.7271).

Serum CgB level of all type 2 diabetes patients did not differ from those of their matched controls (p = 0.1698). The subgroup of type 2 diabetes patients with intensive conservative insulin treatment (n = 34, CgB: 84.87 ± 40.37 ng/mL) had significantly lower serum CgB levels (p = 0.0283), compared to those patients, who were treated with any other regimens of antidiabetic therapies (n = 66, CgB: 107.38 ± 59.74 ng/mL). No correlation could be verified between serum CgA and CgB levels (p = 0.7635).

5. Conclusions

- The present study showed that approximately 20% of the T1DM patients had CgA levels above the upper limit of the normal range.

- In T1DM, a positive correlation can be demonstrated between serum CgA levels and HbA_{1C} levels. Higher serum CgA levels are more often associated with a poorer glycemic control.

- A slight, but steady elevation was observed in CgA level that co-varied with the duration of T1DM: 0.40–1.82% average increase in the serum CgA concentrations can be expected annually.

- Gastric ECL hyperplasia and AIG was significantly more frequent in T1DM patients having high CgA levels.

- More advanced forms of hyperplasia (linear and micronodular hyperplasia) and autoimmune gastritis were found only in T1DM patients of the *High CgA* group.

- ECL hyperplasia stages progressed in most of the T1DM patients with high CgA level, which was also accompanied by a rise in serum CgA levels. CgA levels did not change in those T1DM patients who had a lack of ECL hyperplasia progression.

- The presented case report showed that within a short time period, even a moderate increase in the level of serum CgA should draw the clinicians' attention and may indicate premalignant / malignant lesions of other than the stomach.

- In T2DM, only a few cases were observed where CgA had been increased.

- Serum CgB level was lower in T1DM patients, and in the subgroup of type 2 diabetes patients who received intensive conservative insulin treatment.

We propose that serum CgA should be considered as an auxiliary marker in the routine care of adult diabetes patients. In the case of high CgA further examinations by gastroscopy and imaging techniques should be carried out; and if necessary, an early intervention can be initiated. Based on the results of the present study, the routine measurement of CgA in T2DM would have no practical benefit.

6. Bibliography of the candidate's publications

6.1. Publications related to the dissertation

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