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Serum carbonic anhydrase combined with adiponectin as biomarkers of insulin resistance

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ABSTRACT: Insulin resistance is the main cause of type 2 diabetes. The hyperinsulinemic-euglycemic clamp is a gold standard method for determination of the insulin resistance. However, the test cannot directly measure the insulin resistance, and it is time consuming, costly, labor-intensive and requires an experienced operator. This study aims to develop the protein marker for insulin resistance. Two serum proteins, i.e. carbonic anhydrase and adiponectin were determined by using sandwich enzyme-linked immunosorbent assay (ELISA) in 3 groups of volunteers' sera: healthy group ($n = 35$), insulin resistance group ($n = 32$) and diabetic group ($n = 32$). We explored the associations between these proteins and the insulin resistance. Adiponectin (cutoff $\leqslant 4.73$ μg/ml, area under the curve [AUC] = 0.644 [0.51, 0.777]) ($p < 0.05$) and carbonic anhydrase (cut off $\geqslant 47$ pg/ml, AUC = 0.65 [0.518, 0.783] ($p < 0.050$) have the potential as the biomarkers for diabetes. When used together, adiponectin at cutoff level $\leqslant 4.96$ μg/ml and carbonic anhydrase at cutoff level $\geqslant 36$ pg/ml have the potential as the biomarkers for insulin resistance (AUC = 0.664 [0.532, 0.796]) ($p < 0.05$). These serum protein levels will be a useful tool to diagnose the insulin resistance in human and to guide the right direction of treatment and the behavior change accordingly.

KEYWORDS: insulin resistance, diabetes, adiponectin, carbonic anhydrase, ELISA

INTRODUCTION

Insulin resistance is a primary cause and an important part of developing type 2 Diabetes Mellitus. Supporting evidences come from the reports showing that the presence of insulin resistance occurs 10–20 years earlier than the onset of disease [1, 2]. The hyperinsulinemic-euglycemic clamp is the gold standard method for determining the insulin resistance. The main limitations of the glucose clamp approach are that it is time consuming, labor intensive, expensive and requires an experienced operator [3, 4]. Eighty percent of type 2 diabetes can be prevented if the occurrence of the insulin resistance could be detected at the early stage [5]. Therefore, identification of the biomarkers that are sensitive and specific for the insulin resistance is important for the screening and the effective prevention of type 2 diabetes [6].

Our previous proteomic study revealed that carbonic anhydrase and adiponectin were found among the group of proteins secreted from induced insulin resistance 3T3-L1 adipocytes by using 1 mM palmitic [7, 8]. These proteins were interesting for further study as the biomarkers of insulin resistance.

The main aim of this study was to determine carbonic anhydrase and adiponectin serum levels in 3 groups of volunteer's sera: the healthy group, the insulin resistance group and the diabetes group. We also evaluated the associations between the serum levels of carbonic anhydrase and adiponectin as the indicators of insulin resistance.

MATERIALS AND METHODS

Study subjects

Male and female subjects, aged from 35–70 years, were divided into 3 groups comprising (1) healthy group, (2) insulin resistance group and (3) diabetes group with the following criteria:

(1) Healthy group: fasting plasma glucose < 100 mg/dl, 75 g oral glucose tolerance test (OGTT) < 140 mg/dl, systolic blood pressure (SBP) = 90–129 mmHg, diastolic blood pressure (DBP) = 60–84 mmHg, body mass index (BMI) < 25 kg/m^2.

(2) Insulin resistance group: fasting plasma glucose 100–125 mg/dl, 75 g OGTT 140–199 mg/dl, SBP > 130 mmHg, DBP > 90 mmHg, BMI > 30 kg/m^2 with risk factors of diabetes.

(3) Diabetic group: fasting plasma glucose > 126 mg/dl, 75 g OGTT > 200 mg/dl with type 2 diabetes diagnosed by a physician.

Exclusion criteria were liver disease and infectious disease. This study was approved by the Ethics Committee of Thammasat University (080/2559). The study subjects underwent a thorough informed consent procedure approved by the Ethics Committee, and all provided the written informed consent.

Clinical assessment and biochemical measurements

Subjects were measured in all parameters: resting blood pressure (mean of 2 measurements), height, weight and waist-hip circumference ratio. Fasting blood samples were collected for measurement of fasting glucose, hemoglobin A1c (HbA1c), triglycerides, total cholesterol, high-density lipoprotein (HDL), cholesterol, creatinine, aspartate aminotransferase (AST), alanine transaminase (ALT), C-peptide, carbonic anhydrase and adiponectin.

Plasma glucose was measured using the enzymatic hexokinase method. HbA1c (National Glycohemoglobin Standardization Program; NGSP) was measured using turbidimetric immunoassay (Furuno CA-800, Japan), and plasma triglycerides, total cholesterol, HDL- cholesterol as well as creatinine were measured using the enzymatic colorimetric methods (Furuno CA-800, Japan). Plasma AST and ALT was measured using kinetic methods (Furuno CA-800, Japan). Serum C-peptide was measured using electrochemiluminescence immunoassay (ECLIA) (cobas e411 immunoassay analyzer, Roche Diagnostics, Germany). Serum adiponectin was measured using an ELISA (Quantikine ELISA human total adiponectin/Acrp30 immunoassay, R&D system, USA), and serum carbonic anhydrase was measured using an ELISA (Quantikine ELISA human carbonic anhydrase immunoassay, R&D system, USA).

For 75 g oral glucose tolerance test, after 8- to 12-h fasting, subjects received a load of 75 g of glucose (20% w/v in water) to be consumed in less than 5 min. Blood samples for the measurement of plasma glucose were collected at 0 time and 2 h post-glucose challenge. All subjects were not allowed to smoke, ingest food or do significant physical activity during the 2h-OGTT.

The insulin resistance index was calculated by the formula of Ohkura et al [9] which was equal to 20/fasting C-peptide (nmol/l) × fasting plasma glucose (mmol/l).

Statistical analyses

Descriptive statistics were used to describe the general characteristics of the data such as mean and median. The inferential statistics were used to test the hypothesis of comparing the sample values as follows: the Scheffé post hoc test of Analysis of Variance (ANOVA) was used to compare statistical differences between the volunteer groups. Factors related to insulin resistance were determined with Pearson's Correlation. Correlation testing with Univariate and Multivariate regression analysis was used for evaluating and comparing the effectiveness of protein as an indicator of insulin resistance. Receiver operating characteristic curve (ROC curve) was used to analyze the sensitivity and specificity versus the cutoff value. All statistical tests were two-tailed at a significance level of 0.05. Analyses were performed with SPSS.

RESULTS

Based on the clinical assessment and the biochemical analysis results, 150 subjects were analyzed and categorized by inclusion and exclusion criteria into 3 groups as follows: (1) healthy group ($n = 35$), (2) insulin resistance group ($n = 32$) and (3) diabetes group ($n = 32$). The comparative analyses of the statistical differences between the 3 groups of volunteers (as shown in Table 1) with the Scheffé post hoc test of ANOVA revealed that the variables were statistical differences such as age, weight, height, body mass index, waist-hip ratio, systolic blood pressure, diastolic blood pressure, pulse (beats per minute; BPM), fasting plasma glucose, 2 h OGTT, HbA1c (%), triglycerides, HDL-cholesterol, ALT, C-peptide and insulin resistance index (ANOVA, $p < 0.05$).

When considering the distribution of 2 protein values (carbonic anhydrase and adiponectin) in 3 groups from the quartile range analysis (interquartile range; IQR) to find the median or median (IQR) (Table 1), it was found that the serum levels of adiponectin were significantly different between the healthy group and the diabetic group. Serum levels of carbonic anhydrase were statistically significant differences between the insulin resistance volunteers and the diabetic volunteers.

Table 1 Clinical assessments and biochemical analysis of the volunteers.

Variable	Healthy ($n = 35$)	IR ($n = 32$)	Diabetes ($n = 32$)	p-value			
				ANOVA	Healthy vs. IR	Healthy vs. DM	DM vs. IR
Age	47.2 ± 10.1	52.78 ± 9.21	52.84 ± 9.61	0.025*	0.066	0.062	1
Sex female/male (% females)	29/6 (82.9%)	28/4 (87.5%)	23/9 (71.9%)	0.264	0.594	0.281	0.120
Smoking	2 (8.3%)	0 (0%)	1 (4.5%)	0.449	0.209	0.603	0.360
Weight (kg)	54.11 ± 5.96	65.26 ± 9.82	68.35 ± 13.58	<0.001*	<0.001*	<0.001*	0.481
Height (cm)	158.4 ± 6.53	158.06 ± 8.16	158.44 ± 9.34	0.979	0.985	1	0.983
BMI	21.55 ± 1.79	26.11 ± 3.2	27.18 ± 4.43	<0.001*	<0.001*	<0.001*	0.431
Waist circumference (cm)	76.5 ± 7.7	85.11 ± 8.56	89.37 ± 11.85	<0.001*	0.002*	<0.001*	0.205
Hip circumference (cm)	92.36 ± 10.06	100.25 ± 7.91	100.73 ± 12.11	0.001*	0.008*	0.005*	0.983
Waist-hip ratio	0.83 ± 0.08	0.85 ± 0.06	0.89 ± 0.07	0.006*	0.632	0.007*	0.089
Systolic blood pressure (mmHg)	112.63 ± 10.38	125.47 ± 14.87	136.66 ± 17.97	<0.001*	0.002*	<0.001*	0.011*
Diastolic blood pressure (mmHg)	71.71 ± 9.14	78.84 ± 10.38	81.53 ± 13.32	0.001*	0.034*	0.002*	0.623
Pulse (BPM)	77.15 ± 9.99	80.83 ± 9.11	84.55 ± 12.64	0.026*	0.396	0.026*	0.416
Fasting plasma glucose (mg/dl)	86.86 ± 7.5	108.63 ± 15.97	171.56 ± 75.1	<0.001*	0.133	<0.001*	<0.001*
Glucose 2 h (75 g OGTT) (mg/dl)	89.57 ± 14.98	131.44 ± 32.46	258.0 ± 46.61	<0.001*	<0.001*	<0.001*	<0.001*
HbA1c (%)	5.95 ± 0.24	6.67 ± 0.88	8.23 ± 1.55	<0.001*	0.019*	<0.001*	<0.001*
Creatinine (mg/dl)	0.84 ± 0.14	0.85 ± 0.16	1.08 ± 1.36	0.395	0.999	0.478	0.512
Total cholesterol (mg/dl)	226.91 ± 34.76	226.97 ± 52.42	210.41 ± 54.58	0.277	1	0.372	0.385
Triglycerides (mg/dl)	108.94 ± 50.68	155.56 ± 116.06	177.84 ± 80.2	0.005*	0.089	0.006*	0.584
HDL-cholesterol (mg/dl)	73.60 ± 14.24	66.16 ± 16.85	61.22 ± 11.37	0.003*	0.110	0.003*	0.390
LDL-cholesterol (mg/dl)	131.54 ± 32.08	129.72 ± 44.37	113.72 ± 44.97	0.155	0.983	0.206	0.294
AST (U/L)	21.6 ± 5.94	25.13 ± 8.8	23.75 ± 9.12	0.197	0.205	0.551	0.791
ALT (U/L)	17.69 ± 7.69	25.47 ± 12.94	27.16 ± 14.34	0.003*	0.032*	0.007*	0.851
Adiponectin (μg/ml)	5.86 ± 3.69	5.19 ± 3.20	5.32 ± 7.3	0.844	0.862	0.907	0.995
Median (IQR)	5.2(2.55,7.94)	4.4(3.15,7.53)	2.5(1.40,7.05)	0.070	0.547	0.034*	0.075
Carbonic anhydrase (pg/ml)	83.19 ± 89.81	78.50 ± 67.78	105.68 ± 86.25	0.368	0.973	0.536	0.419
Median (IQR)	53(29.4,110.5)	50.9(36.63,83.29)	75.4(54.3,104.2)	0.091	0.935	0.074	0.044*
C-peptide (ng/ml)	1.60 ± 0.46	2.29 ± 0.91	3.13 ± 1.82	<0.001*	0.065	<0.001*	0.022*
HOMA-IR (Ohkura T [9])	8.56 ± 2.97	5.06 ± 1.93	2.90 ± 1.47	<0.001*	<0.001*	<0.001*	0.001*

Mean ± standard deviation, Scheffé post hoc test of ANOVA. *$p < 0.05$ and $p < 0.001$; IR, insulin resistance; BMI, body mass index; OGTT, oral glucose tolerance test; HbA1c, hemoglobin A1c; HDL, high-density lipoprotein; LDL, low-density lipoprotein; AST, aspartate aminotransferase; ALT, alanine transaminase; IQR, interquartile range; and HOMA-IR, homeostatic model assessment of insulin resistance.

Association between various variables and the insulin resistance index

The relationship between various variables in relation to the insulin resistance and the insulin resistance index calculated according to the formula of Ohkura et al [9] as mentioned in Materials and Methods. To find the factors related to the insulin resistance with Pearson's Correlation, it was found that the variables with the statistical significance were age, weight, body mass index, waist-hip ratio, systolic blood pressure, diastolic blood pressure, pulse (BPM), fasting plasma glucose, 2 h OGTT, HbA1c (%), triglycerides, HDL-cholesterol, ALT, C-peptide and adiponectin < 4.73 μg/ml ($p < 0.05$) (Table 2).

A representative from the group of variables that were statistically significantly associated including age, BMI, systolic pressure, triglycerides, ALT and adiponectin levels lower than 4.73 μg/ml was tested for correlation with the insulin resistance index with Univariate and Multivariate regression analysis, and it was found that the factor that was still related to the insulin resistance index was the body mass index and ALT (Table 3).

Evaluation of the efficacy of carbonic anhydrase and adiponectin levels to indicate insulin resistance and diabetes

The sensitivity and specificity analyses of carbonic anhydrase and adiponectin levels to predict the insulin resistance and the diabetes are shown in Table 4. According to the ROC curve analysis using the 2 protein levels together, the potential for prediction of the insulin resistance and the diabetes could be increased as follows: when using the serum adiponectin level ⩽ 4.96 μg/ml together with the serum carbonic anhydrase level ⩾ 36 pg/ml, the insulin resistance prediction area increased to 0.664 (0.532, 0.796) ($p < 0.05$). When using the serum adiponectin level ⩽ 4.73 μg/ml together with the serum carbonic anhydrase level ⩾ 47 pg/ml, the area of the diabetes prediction increased to 0.744 (0.623, 0.866) ($p < 0.05$) (Table 5).

Table 2 Clinical assessments and biochemical analysis of the volunteers.

Correlation	Insulin resistance index (Ohkura et al [9])	
	r	*p*-value
Age	−0.258	0.010*
Sex female/male (% females)	−0.049	0.631
Smoking	−0.442	< 0.001*
Weight (kg)	0.063	0.536
Height (cm)	−0.542	< 0.001*
BMI	−0.445	< 0.001*
Waist circumference (cm)	−0.356	< 0.001*
Hip circumference (cm)	−0.191	0.058
Waist-hip ratio	−0.438	< 0.001*
Systolic blood pressure (mmHg)	−0.354	< 0.001*
Diastolic blood pressure (mmHg)	−0.303	0.003*
Pulse (BPM)	0.141	0.268
Fasting plasma glucose (mg/dl)	−0.559	< 0.001*
Glucose 2 h (75 g OGTT) (mg/dl)	−0.547	< 0.001*
HbA1c (%)	−0.526	< 0.001*
Creatinine (mg/dl)	−0.151	0.136
Total cholesterol (mg/dl)	−0.006	0.955
Triglycerides (mg/dl)	−0.398	< 0.001*
HDL-cholesterol (mg/dl)	0.263	0.009*
LDL-cholesterol (mg/dl)	0.070	0.488
AST (U/l)	−0.090	0.375
ALT (U/l)	−0.403	< 0.001*
Adiponectin (μg/ml)	0.160	0.113
Adiponectin_low 4.73	−0.324	0.001*
Carbonic anhydrase (pg/ml)	−0.115	0.256
Carbonic anhydrase_up 85	−0.141	0.163
C-peptide (ng/ml)	−0.681	< 0.001*

* $p < 0.05$ and $p < 0.001$; BMI, body mass index; OGTT, oral glucose tolerance test; HbA1c, hemoglobin A1c; HDL, high-density lipoprotein; LDL, low-density lipoprotein; AST, aspartate aminotransferase; and ALT, alanine transaminase.

DISCUSSION

Higher incidence of diabetes has been reported in many countries [10]. Availability of the screening method and the prevention would raise awareness of the patients before developing the disease.

There is evidence that the obese people have a higher risk of developing diabetes than the normal people. One factor is probably due to an increased fatty acid from the digestion of triglycerides from the visceral fat [11]. It was found that palmitic acid can induce insulin resistance [12, 13]. Our previous studies in 2013 reported the proteins expressed in the insulin resistance 3T3-L1 adipocytes induced with palmitic acid by using in-gel digestion coupled with mass spectrometric (GeLC-MS/MS) technique [7]. This led to the selection of the interesting proteins (carbonic anhydrase and adiponectin) in this study to be used as the biomarker for insulin resistance. Carbonic anhydrase is found to be involved in the metabolic process in the glucose biosynthesis. The association of carbonic anhydrase with insulin secretion has been reported which may be linked to the regulation of insulin secretion. Adiponectin is reported to be associated with insulin resistance and can be predicted for the occurrence of diabetes and cardiovascular disease [14].

Analysis of adiponectin and carbonic anhydrase protein levels in the healthy group, the insulin resistance group and the diabetic group showed that the adiponectin protein levels had the following median values (IQR): 5.21 (2.55, 7.94), 4.4 (3.15, 7.53) and 2.53 (1.4, 7.05), respectively. The data showed that the levels of adiponectin protein tended to decrease with the insulin resistance and the diabetes. The carbonic anhydrase protein levels of the healthy group, the insulin resistance group and the diabetic group had the following median values (IQR): 53 (29.36, 110.5), 50.91 (36.63, 83.29) and 75.43 (54.3, 104.25), respectively. These revealed that the levels of carbonic anhydrase protein tend to increase when the patients have insulin resistance and the diabetes.

When testing for the statistically significant differences in the protein levels among the subjects, the adiponectin protein levels were significantly different between the healthy group and the diabetic group. Carbonic anhydrase protein levels were significantly different between the insulin resistance group and the diabetes group. Additionally, it was found that the adiponectin protein level lower than 4.73 μg/ml had a statistically significant association with the insulin resistance index [9].

Adiponectin belongs to the adipocytokine group which plays an important role in controlling the metabolism of glucose and fat. It has an important role in increasing insulin sensitivity both in the muscle and the liver. Adiponectin has a structure consisting of 230 amino acids, approximately 25–30 kDa, made out of fat cells in particular [15, 16]. According to a study conducted by Hu et al [15] in 1996, the size of the fat cells affects the insulin sensitivity in overweight people with type 2 diabetes, meaning that the changes in the levels of the free fatty acid, leptin, resistin and tumor necrosis factor (TNF)-α are very high, but the amount of adiponectin in the bloodstream is significantly reduced. When compared to normal people with the same age and sex, changes of these adipocytokines, especially the decrease in the amount of adiponectin in the bloodstream, affect the increase in the insulin resistance in the patients with diabetes [17, 18]. Consistent with the findings from this research, the level of adiponectin tends to decrease in the insulin resistance group and the diabetes group which is contrary to the increased

Table 3 Univariate and multivariate regression analysis of the volunteers.

	Univariate		Multivariate	
	Crude beta (95% CI)	p-value	Adjusted beta (95% CI)	p-value
Age	−0.084 (−0.148, −0.021)	0.010*	−0.050 (−0.103, 0.004)	0.067
BMI	−0.430 (−0.565, −0.296)	< 0.001*	−0.240 (−0.393, −0.087)	0.002*
Systolic blood pressure (mmHg)	−0.081 (−0.114, −0.047)	< 0.001*	−0.029 (−0.063, 0.005)	0.094
Triglycerides (mg/dl)	−0.014 (−0.021, −0.008)	< 0.001*	−0.006 (−0.012, 0)	0.069
ALT (U/l)	−0.105 (−0.153, −0.057)	< 0.001*	−0.048 (−0.092, −0.004)	0.035*
Adiponectin < 4.73 μg/ml	−2.103 (−3.368, −0.864)	< 0.001*	−0.622 (−1.781, 0.536)	0.289

* $p < 0.05$ and $p < 0.001$; BMI, body mass index; ALT, alanine transaminase; and CI, confidence interval.

Table 4 The sensitivity and specificity analysis of carbonic anhydrase and adiponectin level to predict insulin resistance and diabetes.

Variable	Cutoff	Sensitivity	Specificity	PPV	NPV	Accuracy	LR+	LR−	p-value
Insulin resistance									
Adiponectin (μg/ml)	⩽ 4.96	65.6%	57.1%	58.3%	64.5%	61.2%	1.53	0.60	0.062
Carbonic anhydrase (pg/ml)	⩾ 36	81.3%	34.3%	53.1%	66.7%	56.7%	1.24	0.55	0.152
Diabetes									
Adiponectin (μg/ml)	⩽ 4.73	68.8%	60.0%	61.1%	67.7%	64.2%	1.72	0.52	0.018*
Carbonic anhydrase (pg/ml)	⩾ 47	84.4%	45.7%	58.7%	76.2%	64.2%	1.55	0.34	0.008*

* $p < 0.05$ and $p < 0.001$; PPV, positive predictive value; NPV, negative predictive value; LR+, positive likelihood ratio; and LR−, negative likelihood ratio.

body mass index.

Carbonic anhydrase (CAs, EC 4.2.1.1) is found in most organisms. More than 14 isoforms are found in the cytosol and mitochondria membranes and are secreted in the saliva. The protein plays a role as an enzyme that controls the pH level in most tissues. In addition, it is found to be responsible for the metabolic process in the glucose biosynthesis. This study found an association between the carbonic anhydrase protein and the insulin secretion which may be linked to the regulation of the secretion. Changes in the carbonic anhydrase levels are associated with the metabolic changes, especially in the diabetes patients. Carbonic anhydrase is considered an enzyme that has been studied extensively and linked to various diseases with the change in its catalytic activity. This has interesting clinical applications [19, 20] such as carbonic anhydrase treatment and prevention of obesity [21, 22]. Carbonic anhydrase is being studied as an indicator in the early stages of diabetes testing for detection of insulin resistance [23, 24], consistent with this research. It was found that increased carbonic anhydrase levels could indicate diabetes. The previous research analyzed the level of carbonic anhydrase in red blood cells only in 2 groups of volunteers, which are healthy group and diabetic group [23]. However, in this research, the subjects were divided into 3 groups with the insulin resistance group added, in which the representative results of the analyses revealed more clearly and precisely.

Evaluation of the efficacy of carbonic anhydrase and adiponectin protein levels for determination of the insulin resistance and the diabetes showed that

Table 5 Receiver operating characteristic (ROC) curve analysis for the prediction of insulin resistance and diabetes.

	Insulin resistance		Diabetes	
	AUC (95% CI)	p-value	AUC (95% CI)	p-value
Adiponectin (μg/ml)	0.614 (0.478, 0.749)	0.110	0.644 (0.510, 0.777)	0.043*
Carbonic anhydrase (pg/ml)	0.578 (0.440, 0.715)	0.275	0.650 (0.518, 0.783)	0.034*
Adiponectin and carbonic anhydrase	0.664 (0.532, 0.796)	0.021*	0.744 (0.623, 0.866)	0.001*

* $p < 0.05$ and $p < 0.001$; AUC, area under the curve; and CI, confidence interval.

the 2 proteins have the potentials to identify the diabetes as well as insulin resistance when used together.

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