Association of serum levels of antibodies against ALDOA and FH4 with transient ischemic attack and cerebral infarction

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Abstract

Background and Purpose: Ischemic stroke, such as Transient ischemic attack (TIA) and cerebral infarction (CI) , are the serious problems in the aging society. Therefore, development of biomarkers for TIA and CI is attempted.

Methods: Candidate antigens recognized by IgG autoantibodies in the sera of nineteen TIA patients were screened by a human aortic endothelial cell cDNA library. Serum antibody levels against the antigens were examined by amplified luminescent proximity homogeneous assay-linked immunosorbent assay (AlphaLISA) in healthy donor (HD), TIA, and CI cohorts (n = 285, 92 and 529). A case-control study nested within the Japan Public Health Center-based Prospective Cohort Study (JPHC) was performed.

Results: Aldolase A, fructose-bisphosphate (ALDOA) and fumarate hydratase (FH) were identified as the candidate antigens. AlphaLISA revealed that anti-ALDOA and anti-FH antibody levels were both higher in TIA or CI patients than in HDs ($P < 0.0001$). The levels of anti-ALDOA [Odds ratio (OR): 2.46, $P = 0.005$] and anti-FH (OR: 2.49, $P = 0.0037$) were independent predictors of TIA by multivariate logistic regression analysis, similar results were found in CI. The case-control study showed the levels of anti-ALDOA (OR: 2.50, $P < 0.01$) and anti-FH (OR: 2.60, $P < 0.01$) were associated with risk of CI. Spearman’s correlation analysis demonstrated an association between the anti-ALDOA and anti-FH levels and risk factors of ischemic stroke, such as age, smoking habit, coronary heart disease, and hypertension.

Conclusions: Anti-ALDOA and anti-FH antibodies can serve as novel potential biomarkers for prediction of TIA and CI.

Background

Ischemic stroke, such as Transient ischemic attack (TIA) and cerebral infarction (CI), are the most common cerebrovascular disorders worldwide. TIA is a transient episode of neurological dysfunction caused by focal brain, spinal cord, or retinal ischemia, without acute infarction [1]. CI is an episode of neurological dysfunction caused by focal brain infarction, which is a major cause of fatality and disability [2]. Patients with TIA are at high risk of CI. Epidemiologic studies revealed that the

prevalence of prior TIA ranged from 15–30% among patients who present with CI. Additionally, the risk of CI on the 7th, 30th, and 90th-day post-TIA was 2.0–8.0%, 8.0–13.5%, and 9.5–20.1%, respectively [3, 4]. TIA with progressive aggravation represents an early warning signal for CI. Therefore, the key to reduce ischemic stroke's influence on human health is early diagnosing TIA and predicting the onset of CI [5].

To date, there are many means of early prediction for TIA and CI in the medical field, including modern imaging techniques (e.g., transcranial Doppler [6], computed tomography, magnetic resonance imaging [7], cerebral angiography [8]); blood biochemical indicators (e.g., oxidatively modified low-density lipoprotein [9], homocysteine [10], lipoprotein-related phospholipase A2, C-reactive protein [11], heat shock protein [12]); and comprehensive assessment of risk factors [13] (e.g., hypertension (HT), hyperlipidemia, body mass index (BMI), obesity, smoking habits, family history). However, the above-mentioned methods are frequently insufficient to represent standard approaches for early diagnosis of TIA and prediction the onset of CI. Therefore, it is usually expected to find novel biomarkers that would largely improve the management and prognosis of such patients [14].

It is well documented that atherosclerosis is highly likely to play a key role in the pathogenesis of ischemic stroke, and most incident ischemic stroke (i.e. TIA and CI) are based on the atherosclerosis [15]. Atherosclerosis is not only a simple pathological process of lipid deposition in the vascular wall. However, the most recent accomplishments have indicated that atherosclerosis is an inflammatory proliferative dynamic mechanism induced by an excessive autoimmune response following the injury of vascular endothelial cells and smooth muscle cells [16]. Endogenous antigens cause autoimmune responses significantly influencing the development process of atherosclerosis, which ultimately leads to narrowing or blockage of the offending artery [17]. Of note: autoantibodies induced by the antigens have been detected in the sera of patients with atherosclerosis-related diseases, such as CI, coronary artery disease (CAD) and diabetes mellitus (DM) [18].

An established method for identifying endogenous antigenic proteins is the serological identification of antigens by recombinant cDNA expression cloning (SEREX), which represents a combination of

molecular cloning and serological typing by using phage expression libraries [19]. This method was originally developed to screen out tumor-associated antigens, which has been used to identify more than 2,300 novel tumor antigens in a public access online database known as the Cancer Immunome Database (CID) [20, 21]. And so, it is considered one of the most effective methods for the identification of antigenic targets on a genome scale [22–30]. As a result, it has also been used for autoimmune diseases, such as systemic lupus erythematosus, Kawasaki disease, Bechet's disease, and multiple sclerosis in the recent years [22–25]. In earlier studies, we used SEREX for atherosclerosis-related diseases and for the identification of antibodies against RPA2 [26], MMP1, CBX1 and CBX5 [27] in CI, and ATP2B4, BMP-1 [28], TUBB2C [29] and SH3BP5 [30] in other atherosclerosis-related diseases.

Both TIA and CI have the pathological basis of atherosclerosis [15], and we found that atherosclerosis can cause the increase of serum autoantibody level in the early stage of lesions through SEREX [26–30]. Based on this background, it is clear that there is a significant need for the identification of sensitive, specific, and novel biomarkers to early predicting of TIA and CI. In the present study, our goal was to identify autoantibodies associated with TIA and CI by SEREX, which could be used as molecular predicted biomarkers to reflect the status of disease.

Methods

Ethics statement

This study was approved by both the Local Ethical Review Board of the Graduate School of Medicine, Chiba University, and by the Ethical Review Board of co-operating universities, hospitals, and research institutes. We performed the study in accordance with the principles of the Declaration of Helsinki. Recombinant DNA studies were performed with the official permission of the Graduate School of Medicine, Chiba University. The latter experiments were conducted in conformity with the rules of the Japanese government. We obtained informed consent from all participants for all the studies.

Sera of patients and healthy donors (HDs)

We collected serum samples from healthy donors (HDs) and patients diagnosed with TIA and CI caused by atherosclerotic vulnerable plaque [31, 32]. HDs were selected from individuals who did not have a history of TIA and CI, including acute-phase cerebral infarction (aCI) or old-cerebral infarction

(oCI). Additionally, all of the selected HDs underwent medical checkups, including cerebral MRI. Subjects with autoimmune disease were excluded. A total of 19 TIA patients were randomly selected for immunological screening by SEREX. In order to perform a comparison of the serum antibody levels, we set up four independent groups, which included 621 patients and 285 HDs. Of the 621 patients assigned to the validated disease groups, 92, 464, and 65 suffered from TIA, aCI, and oCI, respectively. Table 1 shows the baseline characteristics of participants.

Table 1

Baseline characteristics of subjects enrolled in the study.

	SEREX	AlphaLISA			
TIA (n = 19)	Stroke			HD (n = 285)	
	TIA (n = 92)	aCI (n = 464)	oCI (n = 65)		
Age	68.3*** (± 10.2)	70.2*** (± 11.6)	75.5*** (± 11.5)	73.3*** (± 9.2)	52.3 (± 11.7)
Male gender	16 (84.2%)	55 (59.7%)	271 (58.4%)	48 (73.8%)	188 (65.9%)
HT	13*** (68.4%)	60*** (65.2%)	335*** (72.2%)	53*** (81.5%)	57 (20.0%)
DM	3*** (15.8%)	26*** (28.3%)	125*** (26.9%)	22*** (33.8%)	11 (3.9%)
Hyperlipidemia	3 (15.8%)	36*** (39.1%)	122*** (26.3%)	25*** (38.5%)	40 (14.0%)
CHD	1*** (5.2%)	5*** (5.4%)	40*** (8.6%)	2*** (3.1%)	0
Obesity (BMI ≥ 25)	10 (52.6%)	30 (32.6%)	127 (27.4%)	11 (16.9%)	88 (30.9%)
Smoking	12 (63.1%)	43 (46.7%)	228 (49.1%)	33 (50.8%)	132 (46.3%)

Data represents means (± SD) for continuous data and n (%) for categorical data.

*** $P < 0.001$ vs. HD.

TIA, transient ischemic attack; aCI, acute cerebral infarction; HD, healthy donor; oCI, old cerebral infarction; HT, hypertension; DM, diabetes mellitus; CHD, coronary heart disease.

We obtained sera of patients with TIA, aCI and oCI from Chiba Prefectural Sawara Hospital, Chiba Rosai Hospital, and Chiba Aoba Municipal Hospital. Additionally, we obtained sera of HDs from Chiba Prefectural Sawara Hospital, Higashi Funabashi Hospital, and Port Square Kashiwado Clinic. Following collection, samples were centrifuged at 3,000 g for 10 min at room temperature, and supernatants were stored at – 80 °C until use. We avoided repeated thawing and freezing of samples.

Clinical data

From patients' clinical records, we collected data regarding the risk factors for atherosclerosis, including the following: age, gender, hypertension (HT), diabetes mellitus (DM), hyperlipidemia, coronary heart disease (CHD), obesity, and smoking. HT was defined as a history of systolic blood pressure > 140 mmHg, diastolic blood pressure > 90 mmHg, or the use of antihypertensive agents. DM was defined as having previously diagnosed DM and/or DM treated with medication and/or fasting

blood glucose ≥ 126 mg/dL. Hyperlipidemia was defined as a history of total cholesterol > 220 mg/dL, triglycerides > 150 mg/dL, or use of lipid-lowering agents. CHD was defined as a history of myocardial infarction or angina pectoris. Patients were considered as smokers if they either smoked during the study period or had a history of smoking. Finally, obesity was defined as BMI ≥ 25. Additionally, we collected the participants' serum routine examination results, including blood routine, serum biochemistry, blood electrolytes, etc.

Screening by expression cloning

We performed immunoscreening by using a modified version of previously published methods [18, 26, 30, 33, 34]. In order to screen for clones that were immunoreactive against sera of patients with TIA, we used a commercially available human aortic endothelial cell cDNA library (Uni-ZAP XR Premade Library, Stratagene, La Jolla, CA). Escherichia coli (E. coli) XL1-Blue MRF' was infected with Uni-ZAP XR phage. The expression of resident cDNA clones was induced after blotting infected bacteria onto nitrocellulose membranes (NitroBind, Osmonics, Minnetonka, MN), which were pretreated with 10 mM isopropyl-β-D-thiogalactoside (IPTG) (Wako Pure Chemicals, Osaka, Japan) for 30 min. Membranes with bacterial proteins were washed three times with TBS-T [20 mM Tris-HCl (pH 7.5), 0.15 M NaCl, and 0.05% Tween-20], Subsequently, we blocked nonspecific binding by incubating membranes with 1% protease-free bovine serum albumin (Nacalai Tesque, Inc., Kyoto, Japan) in TBS-T for 1 h. Overnight incubation with 1 : 2000 diluted sera of patients was performed on the membranes. Following three washes with TBS-T, membranes were incubated for 1 h with 1:5000 diluted alkaline phosphatase-conjugated goat anti-human IgG (Jackson ImmunReseach Laboratories, West Grove, PA). We visualized positive reactions by incubating membranes in a color development solution [100 mM Tris-HCl (pH 9.5), 100 mM NaCl, and 5 mM $MgCl_2$]. The solution contained 0.15 mg/mL of 5-bromo-4-chloro-3-indolylphospate (Wako Pure Chemicals) and 0.3 mg/mL of nitro blue tetrazolium (Wako Pure Chemicals). To obtain monoclonality, positive clones were re-cloned for two additional times, as previously described [18, 30, 34].

Sequence analysis of identified antigens

We converted the monoclonalized phage cDNA clones to pBluescript phagemids by in vivo excision

using ExAssist helper phage (Stratagene). Plasmid DNA was obtained from the E. coli SOLR strains transformed by the phagemids. Following sequencing of inserted cDNAs, homologous analysis was performed using a public database provided by the National Center for Biotechnology Information (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Construction of expression vectors

We constructed expression plasmids of glutathione-S-transferase (GST)-fused proteins by recombining the cDNA sequences into pGEX-4T vectors (GE Healthcare Life Sciences, Pittsburgh, PA), as previously described [6, 17, 21, 33, 34]. EcoRI and XhoI digestion was performed on the pBluescript plasmids containing cDNA inserts. Digestion products were then separated via agarose gel electrophoresis. Inserted cDNA fragments were isolated using GenElute™ Minus EtBr Spin Columns (Merck, Darmstadt, Germany). Subsequently, ligation was performed in frame to EcoRI- and XhoI-digested pGEX- 4T-2 linearized vectors using a Ligation-Convenience Kit (Nippon Gene, Toyama, Japan). Ligation mixtures were then used to transform ECOS^{TM}-competent E. coli BL-21 (Nippon Gene).

Purification of recombinant candidate proteins

Transformed E. coli BL-21 cells containing pGEX-4T-2 clones were cultured in 200 mL of Luria broth and treated with 0.1 mM IPTG for 3 h. The IPTG-treated cells were processed as follows: harvested, washed with phosphate-buffered saline, and lysed by sonication in BugBuster Master Mix (Novagen, San Diego, CA). Subsequently, cell lysates were centrifuged at 13,000 g for 10 min at 4 °C. The GST fusion recombinant proteins recovered in the supernatant fraction were directly affinity purified by glutathione-Sepharose column chromatography (GE Healthcare Life Sciences), according to the manufacturer’s instructions. The purified proteins were then concentrated using Amicon Ultra-15 Centrifugal Filter Devices (Merck Millipore, Darmstadt, Germany), as previously described [26, 28, 30].

We dissolved the precipitates containing recombinant proteins in 8 M urea in TED buffer [50 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 1 mM dithiothreitol]. Subsequently, we performed dialysis stepwise against 4 and 2 M urea in TED buffer for 1 h each. Samples were then dialyzed against TED buffer for $>$ 12 h and centrifuged at 10,000 g for 30 min at 4 °C. We purified the recombinant proteins

recovered in the supernatant using glutathione-Sepharose, as described above.

Western blotting

GST, GST-ALDOA, and GST-FH proteins (0.3 μg) were electrophoresed through SDS-polyacrylamide gel followed by Western blotting. To this end, we used anti-GST (goat) (Rockland, Gilbertsville, PA) or 1:5000-diluted sera from patients with TIA and CI (#350, and #692). Proteins were then incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (donkey anti-goat or anti-human IgG, Santa Cruz Biotechnology, CA). Subsequently, we detected immunoreactivity with the Immobilon™ Western HRP Substrate (Merck KGaA, Darmstadt, Germany), as previously described [30, 33, 35, 36].

Amplified luminescent proximity homogeneous assay-linked immunosorbent assay (AlphaLISA) of antibody biomarkers

Amplified luminescent proximity homogeneous assay-linked immunosorbent assay (AlphaLISA) was applied to the quantitative measurement of serum antibodies against the purified proteins. It was performed using 384-well microtiter plates (white opaque OptiPlateTM, Perkin Elmer, Waltham, MA) containing 2.5 μl of 1:100 diluted sera and 2.5 μl of GST or 10 μg/ml GST fusion proteins in AlphaLISA buffer [25 mM HEPES (pH 7.4), 0.1% casein, 0.5% Triton X-100, 1 mg/ml dextran-500, and 0.05% Proclin-300]. We incubated the reaction mixture at room temperature for 6–8 h. We subsequently added anti-human IgG-conjugated acceptor beads (2.5 μL, 40 μg/mL) and glutathione-conjugated donor beads (2.5 μL, 40 μg/mL). The mixture was then incubated for 14 d at room temperature in the dark. Reading of the chemical emission was performed on an EnSpire Alpha microplate reader (Perkin Elmer), as previously described [27, 30, 34]. We calculated specific reactions by subtracting the Alpha values (Alpha counts) of the GST control from those of GST fusion proteins.

Nested case-control study

A nested case-cohort study was performed using the above AlphaLISA detection antibody levels. The study nested within the Japan Public Health Center-based Prospective Study [37, 38]. It involved approximately 30,000 Japanese individuals aged 40–69 years, whose serum was stored between 1991 and 1993. ALDOA and FH’s antibody levels were measured in 202 cases of incident CI developed in patients of the cohort between the baseline and 2008, and in 202 controls with matching age, gender,

and area.

Statistical analyses

We compared differences in the Alpha values between two groups by using Student's t-test and Mann-Whitney U test. Additionally, we determined the correlation between Alpha values and clinical case data using Spearman's correlation analysis. In order to identify the set of variables that could be used to classify participants according to positive history for ischemic stroke, univariate and multivariate logistic regression analyses were used. Conditional logistic regression model was used to estimate the odds ratios (ORs) for cerebral infarction compared to the antibody levels of ALDOA and FH in the nested case-control study. We assessed the predictive values of markers for diseases by receiver operating characteristic (ROC) analysis. Additionally, the cutoff values were set to maximize the sum of sensitivity and specificity. All tests were two-tailed. We considered as statistically significant $P < 0.05$. All statistical analyses were performed using either the SPSS 13.0 software (SPSS Inc., Chicago, IL) or GraphPad Prism 5 (GraphPad Software, La Jolla, CA).

Results

Identification of ALDOA and FH as antigens recognized by sera of patients with TIA

We observed two independent clones in the sera of 19 TIA patients by expression cloning (Fig. 1). Specifically, we found a sequence homology with aldolase, fructose-bisphosphate A (ALDOA) (Accession number: NM_184041) and fumarate hydratase (FH) (Accession number: NM_000143), respectively. The region of ALDOA between amino acids 70 and 469 was obtained as a pBluescript II clone and then recombined into a pGEX-4T-2 expression vector. Similarly, the cloned region of FH between amino acids 1 and 185, and was recombined into pGEX-4T-2 vectors. Recombinant ALDOA and FH proteins were expressed in E. coli as GST fusion proteins. They were subsequently purified by affinity chromatography using glutathione-Sepharose.

Presence of serum antibodies was confirmed by Western blotting

We aimed at confirming the presence of anti-ALDOA (ALDOA-Abs) and anti-FH (FH-Abs) antibodies in sera. To this end, Western blotting was performed using sera obtained from TIA and CI patients. Using an anti-GST antibody, GST- ALDOA, GST- FH, and GST proteins were recognized as reactions of 65-kDa, 67-kDa, and 28-kDa proteins, respectively (Fig. 2). On the contrary, GST-ALDOA and/or GST-FH,

but not GST, reacted with serum antibodies of patients #350 and #692. Based on these findings, we suggest that most, if not all, of GST fusion antigen proteins' reactivity with serum antibodies, may be due to antigen proteins and not the GST domain. In the present study, specific reactions against ALDOA or FH proteins were estimated by antibody levels toward GST-tagged antigen proteins subtracted by the levels toward GST.

Levels of ALDOA-Abs and FH-Abs are increased in patients with TIA and CI

In order to quantitatively analyze ALDOA-Abs and FH-Abs levels in serum, AlphaLISA was used. To this end, we examined the sera of HDs and patients with TIA, aCI, or oCI. The Alpha counts represent the luminescent photon counts corresponding to the antibody levels. Our results showed that the levels of ALDOA-Abs and FH-Abs in the three ischemic cerebrovascular diseases (i.e., TIA, aCI, and oCI) were significantly higher vs. HDs ($P < 0.05$) (Table 2). Furthermore, we observed no significant difference in the Alpha counts among patients with the three ischemic cerebrovascular diseases (Fig. 3). Therefore, ALDOA-Abs and FH-Abs levels may be closely related to ischemic cerebrovascular diseases, but not related to the disease type.

Table 2
Comparison of serum antibody levels between HDs, TIA, aCI and oCI patients examined by AlphaLISA.

	HD			TIA			aCI			oCI	
	ALDOA	FH		ALDOA	FH		ALDOA	FH		ALDOA	FH
Average	16,326	2,492		20,675	3,852		20,431	3,850		20,144	4,226
SD	10,410	2,367		12,464	3,765		10,564	3,699		10,633	3,112
Total number	285	285		92	92		464	464		65	65
P (vs. HD)				0.0030	0.0015		2.6E-07	1.5E-09		0.0102	6.2E-05

The average, SD, and the total sample number are presented for HDs and patients as well as P values of statistical comparisons between HDs and patients. We used as antigens purified GST-ALDOA and GST-FH proteins. P values less than 0.05 are marked in bold.

We performed the ROC analysis to evaluate the ability of these markers to detect TIA, aCI, and oCI. The areas under the curve (AUC) of ALDOA-Abs and FH-Abs for TIA were 0.63 [95% confidence interval (CI): 0.56–0.69] (Fig. 4a) and 0.63 (95% CI: 0.56–0.70) (Fig. 4d), respectively. While the AUC for aCI were 0.63 (95% CI: 0.60–0.67) (Fig. 4b) and 0.63 (95% CI: 0.59–0.67) (Fig. 4e), respectively. Additionally, the AUC for oCI were 0.62 (95% CI: 0.54–0.70) (Fig. 4c) and 0.67 (95% CI: 0.60–0.75) (Fig. 4f). At a cutoff value of ALDOA-Ab levels of 14,869, the antibody level's sensitivity and specificity for TIA diagnosis were 69.57% and 54.74%, respectively (Fig. 4a). Such levels were similar to those

for aCI diagnosis (69.40% and 51.58%, respectively) (Fig. 4b and 4c). Furthermore, sensitivity and specificity for FH-Abs are shown in Fig. 4d, 4e, and 4 f.

Association between TIA and clinical parameters including ALDOA-Abs and FH-Abs levels

Results of univariate and multivariate logistic regression analyses are shown in Table 3. Using the cutoff value of 14,869 and 2,849, respectively, univariate logistic regression analysis revealed that the elevated ALDOA-Abs (OR: 2.91, 95% CI: 1.76 − 4.83, $P < 0.0001$) and FH-Abs (OR: 2.88, 95% CI: 1.78–4.67, $P < 0.0001$) level was associated with the increased risk of TIA, respectively. We included in the multivariate analysis factors with a univariate $P < 0.05$. The multivariate logistic regression analysis revealed that elevated ALDOA-Abs (OR: 2.46, 95% CI: 1.31 − 4.62, $P = 0.0050$) and FH-Abs (OR: 2.49, 95% CI: 1.35–4.63, $P = 0.0037$) level was an independent predictor of TIA, respectively. The predictive value of ALDOA-Abs and FH-Abs for TIA was not inferior to other known risk factors of TIA. Specifically, these include the following: age (OR: 6.04, 95% CI: 3.15–11.58, $P < 0.0001$); HT (OR: 2.97, 95% CI: 1.61–5.45, $P = 0.0005$); and DM (OR: 5.31, 95% CI: 2.05–13.79, $P = 0.0006$).

Table 3
Logistic regression of predictive factors for TIA (n = 377; no. of events = 92).

	Univariate			Multivariate		
	P	OR	95% CI	P	OR	95% CI
Age (≥ 60)	0.0000	9.97	5.65–17.59	0.0000	6.04	3.15–11.58
Gender	0.2304	1.34	0.83–2.17			
HT	0.0000	7.5	4.47–2.59	0.0005	2.97	1.61–5.45
DM	0.0000	10.35	4.88–21.94	0.0006	5.31	2.05–13.79
HL	0.0000	3.94	2.30–6.73	0.0523	1.94	0.99–3.79
CHD	0.0132	8.13	1.55–42.66	0.8917	1.14	0.17–7.77
BMI (≥ 25)	0.7768	1.08	0.65–1.8			
Smoking	0.9653	1.01	0.63–1.62			
ALDOA[a]	0.0000	2.91	1.76–4.83	0.0050	2.46	1.31–4.62
FH[b]	0.0000	2.88	1.78–4.67	0.0037	2.49	1.35–4.63

[a] ADOLA, elevated ADOLA-Ab levels. ADOLA -Abs cut off value was 14,869 based on ROC curve analysis.

[b] FH, elevated FH-Ab levels. FH -Abs cut off value was 2,849 based on ROC curve analysis.

HT, hypertension; DM, diabetes mellitus; HL, hyperlipidemia; CHD, coronary heart disease; BMI, Body Mass Index; OR, odds ratio; 95% CI, 95% confidence intervals.

Association between CI and serum antibodies including ALDOA-Abs and FH-Abs

The above logistic regression analysis proved that ALDOA-Abs and FH-Abs are independent early warning risk factors for TIA, and their elevated level can represent the occurrence of TIA. To further validate their association with CI, we conducted a prospective case-control study nested within the Japan Public Health Center-based Prospective Study. Table 4 displays the results. Specifically, we observed that ALDOA-Abs levels were positively and strongly associated with the risk of cerebral

infarction. We found that the ORs (95% CIs) were 2.38 (1.24–4.55) and 2.50 (1.26–4.96) for individuals with the second and highest quartiles of antibody level, respectively, as compared with the lowest quartile. Additionally, we observed that FH-Abs levels were also positively associated with the risk of cerebral infarction. Specifically, we observed that the ORs (95% CIs) were 2.17 (1.20–3.92) and 2.60 (1.41–4.80), for persons with the third and highest quartiles of antibody level, respectively.

Table 4

Age and sex-matched, conditional odds ratios and 95% confidence intervals of incident CI according to antibody markers (202 cases and 202 controls).

Antibody marker		case / control	Matched OR (95% CI)
ALDOA-Abs	1st	30 / 50	1.00
	2nd	62 / 51	**2.38 (1.24–4.55)**
	3rd	50 / 51	1.95 (1.00–3.82)
	4th	60 / 50	**2.50 (1.26–4.96)**
FH-Abs	1st	29 / 50	1.00
	2nd	40 / 51	1.33 (0.72–2.48)
	3rd	62 / 51	**2.17 (1.20–3.92)**
	4th	71 / 50	**2.60 (1.41–4.80)**

OR values more than 2.00 are marked in bold. OR, odds ratio; 95% CI, 95% confidence intervals.

Association between ALDOA-Abs and FH-Abs and other clinical parameters

We then examined the correlation between ALDOA-Abs and FH-Abs' serum levels and other clinical parameters (Table 5). We observed a weak correlation between serum levels of ALDOA-Abs ($r = 0.1973$, $P < 0.0001$) and FH-Abs ($r = 0.2369$, $P < 0.0001$) and age. ALDOA-Abs and FH-Abs levels were both positively correlated with cigarette smoking habits, DM, HT, CHD, and carotid intima-media thickness. Additionally, while ALDOA-Ab levels were positively correlated with DM and negatively correlated with BMI, FH-Ab levels had no correlation with DM and BMI. No significant correlation was observed between serum levels of ALDOA-Abs and FH-Abs and most biochemical indexes ($r < 0.3000$, $P > 0.05$).

Table 5

Correlation analysis between serum antibody marker levels and the indices in HDs and CI patients.

	ALDOA-Abs		FH4-Abs	
	r value	P value	r value	P value
Gender	-0.0386	0.2464	0.0439	0.1866
Age	**0.1973**	**< 0.0001**	**0.2369**	**< 0.0001**
Smoking period	**0.1798**	**< 0.0001**	**0.0880**	**0.0237**
Diabetes mellitus (Complic)	0.0479	0.1500	**0.0820**	**0.0135**
Hypertension (Complic)	**0.1323**	**< 0.0001**	**0.1684**	**< 0.0001**
Blood pressure	**0.1574**	**< 0.0001**	**0.0919**	**0.0196**
Coronary heart disease (Complic)	**0.0772**	**0.0201**	**0.0679**	**0.0411**
Hyperlipidemia (Complic)	-0.0294	0.3770	-0.0307	0.3563
Body mass index	-0.0154	0.6477	-0.1074	0.0014

Body mass index	-0.0154	0.6477	-0.1074	0.0014
Max intima-media thickness	0.2353	< 0.0001	0.2179	< 0.0001
Albumin globulin ratio	-0.1133	0.0040	-0.1107	0.0049
Aspartate transaminase	0.0282	0.4660	0.0236	0.5422
Alanine transaminase	-0.0366	0.3441	-0.0433	0.2631
Alkaline phosphatase	0.0609	0.1319	0.0699	0.0836
Lactate dehydrogenase	0.0634	0.1073	0.0495	0.2082
Total bilirubin	-0.0459	0.2419	-0.0369	0.3460
Cholinesterase	-0.1175	0.0094	-0.1991	< 0.0001
γ- Glutamyl transpeptidase	0.0558	0.1643	-0.0484	0.2274
Total protein	-0.1475	0.0002	-0.1048	0.0076
Albumin	-0.1652	< 0.0001	-0.1453	0.0002
Blood urea nitrogen	0.0340	0.3802	0.0538	0.1647
Creatinin	0.0058	0.8808	0.0146	0.7065
Glomerular filtration rate	-0.0044	0.9131	-0.0556	0.1715
Uric acid	0.0509	0.2716	-0.0233	0.6148
Amylase	-0.0890	0.0817	-0.0336	0.5113
Total cholesterol	-0.1453	0.0005	-0.1262	0.0024
High density lipoprotein cholesterol	-0.0658	0.1855	-0.0503	0.3125
Triglyceride	-0.0904	0.0579	-0.1335	0.0050
Na^+	0.0684	0.0794	-0.0467	0.2316
K^+	-0.0563	0.1495	-0.0217	0.5783
Cl^-	0.0632	0.1052	0.0193	0.6220
C-reactive protein	0.1788	0.0001	0.0491	0.2915
White blood cell number	0.0909	0.0188	0.0737	0.0569
Red blood cell number	-0.0781	0.0436	-0.1152	0.0029
Hemoglobin	-0.0565	0.1447	-0.0984	0.0110
Hematocrit	-0.0506	0.1915	-0.0971	0.0120
Mean corpuscular volume	0.0459	0.2361	0.0542	0.1621
Mean corpuscular hemoglobin	0.0291	0.4530	0.0322	0.4060
Mean corpuscular hemoglobin concentration	-0.0442	0.2538	-0.0543	0.1612
RBC volume distributing width	0.1159	0.0027	0.0598	0.1228
Platelet number	-0.0335	0.3875	-0.0762	0.0490
Mean platelet volume	-0.0032	0.9334	-0.0021	0.9569
Plateletcrit	-0.0323	0.4047	-0.0902	0.0197
Platelet distribution width	-0.0174	0.6534	-0.0382	0.3245
Blood sugar	0.0861	0.0327	0.1512	0.0002
Glycated hemoglobin A1c	-0.0320	0.4758	-0.0183	0.6830
Glycated hemoglobin A1	0.0452	0.5426	0.0527	0.4771

The data on the patients enrolled in the study were obtained as follows. HD subjects were enrolled in Chiba Prefectural Sawara Hospital, Higashi Funabashi Hospital, and Port Square Kashiwado Clinic. TIA, aCI or oCI patients' subjects were enrolled in Chiba Prefectural Sawara Hospital, Chiba Rosai Hospital, and Chiba Aoba Municipal Hospital. We used the Spearman's correlation analysis and multivariate logistic regression analysis to calculate correlation coefficients (r) and P values. In bold we marked $P < 0.05$.

Discussion

The two antigens, ALDOA and FH, were found following serological identification of antigens by means

of recombinant cDNA expression cloning (SEREX). Serum IgG antibodies in TIA patients further recognized them. Additionally, we confirmed by Western blotting the presence of antibodies against ALDOA and FH in the patients' serum (Fig. 2). Furthermore, by means of AlphaLISA, we evaluated the antibody levels. AlphaLISA also allowed us to compare the levels between patients and HDs. Our results show that, compared with HDs, the antibody levels of anti-ALDOA(ALDOA-Abs) and anti-FH(FH-Abs) were significantly elevated in both TIA patients and those with CI (Fig. 3). We gathered additional confirmation that these antibodies are independent predictors of TIA (Table 3). Of note, TIA has a tendency to develop into CI and is a clear CI risk factor [39]. As an independent early warning risk factor for TIA, these elevated antibody levels may also be predictive markers of CI. Therefore, confirmation was obtained by further statistical analysis of clinical data and prospective case-control studies nested in large community-based samples (Tables 3 and 4).

Earlier studies have shown that ALDOA, also known as fructose-bisphosphate aldolase A, represents one of the glycolytic enzymes that catalyze the reversible conversion of fructose-1, 6-bisphosphate to glyceraldehyde-3-phosphate and dihydroxyacetone phosphate [40]. ALDOA is widely distributed in whole body tissues. As a catalytic enzyme, ALDOA represents one of the key enzymes in the glycolysis process. Of note, it plays a role in the hypoxia responses regulating both glucose and energy metabolism and can serve as hypoxia biomarkers [41]. Ischemic stroke represents a typical atherosclerosis-related disease. Its basic pathophysiological feature is represented by local tissue hypoxia. Studies have shown that ALDOA is a hypoxia-inducible gene expression product [42]. When brain tissue undergoes ischemia or hypoxia, brain cells react by stimulating both glucose uptake and metabolism. Their goal is to compensate for the reduction in energy production by inducing overexpression of ALDOA [43]. Hypoxia-inducible factor 1α (HIF-1α) is a transcription factor sensitive to hypoxia-inducible genes. HIF-1α up-regulates ALDOA's expression in hypoxic cells [44], thereby enhancing its glycolysis metabolism. A previous study by Chang et al. proved that ALDOA and HIF-1α were able to co-act, both evoking and enhancing the expression of matrix metalloproteinases [45]. These results are in line with our previous study [27] where we found an increased specificity of anti-MMP1 antibodies in the serum of TIA patients. MMPs can degrade the main components of the

vascular extracellular matrix, which is an important factor responsible for the induction of atherosclerosis. An intimate relationship between ALDOA and MMPs can be observed. We believe that such association proves that ALDOA's overexpression may not only be a sequential pathological process in TIA’s development, but it may also be related to TIA’s initiation and deterioration. Of note, we observed that ALDOA-Abs levels had a constant positive correlation with the following characteristics: age, smoking, HT, obesity (BMI), and CHD (Table 5). ALDOA is silent in the presence of adequate nutritional status (e.g., hyperglycemia, hyperlipidemia, high protein, etc.). As a consequence, ALDOA-Abs are not elevated in the serum of patients with the following conditions: hyperglycemia, DM, hyperlipidemia, and high protein status. These results suggest two explanations. First, that ALDOA-Abs may represent a biomarker of TIA. Second, that ALDOA-Abs represent a marker more specific for atherosclerosis-related diseases, but not associated with glucose tolerance or blood lipids.

FH is a key enzyme involved in the tricarboxylic acid (TCA) cycle. It can reversibly catalyze the conversion of fumaric acid to L-malate in the cells [46]. A primary function of the TCA cycle is the oxidation of pyruvate, supplied by the glycolytic pathway, with the goal of producing energy. In addition to its classical metabolic-related functions, FH has other non-metabolic-related functions under the stimulation of cells [47]. Reports have associated FH with tumorigenesis, specifically by altering the gene expression site and configuration of tumor cells [48]. In an earlier study, Xiao et al. found that FH could antagonize α-ketoglutarate-dependent demethylase through its metabolite fumarate, thereby affecting histone methylation [49]. In addition, Wang et al. also showed that FH exhibited adenosine monophosphate-activated protein kinase-mediated phosphorylation in the absence of glucose or hypoxia, which inhibited histone’s demethylation by lysine-specific demethylase 2A [50]. FH inhibits histone methylation by reducing the physiological activity of vascular endothelial growth factor (VEGF) [51]. This, in turn, affects the repair and remodeling of vascular endothelium following atherosclerosis. It is noteworthy that abnormal histone methylation is responsible for significant gene expression changes, including VEGF. It has been shown that FH plays an important regulatory role in atherosclerosis’ occurrence and development [52]. Based on this

background, our findings can be explained. Specifically, FH-Abs levels in TIA and ischemic stroke patients are significantly higher than those in HDs. FH's metabolic function is related to that of ALDOA, which can provide energy to the body through physiological reactions. However, as opposed to ALDOA, when hyperglycemia occurs, FH's expression is induced by the excited TCA cycle. Therefore, FH is associated not only with common atherosclerotic risk factors (e.g., age, blood pressure, obesity, CHD) but also with DM. In the present study, we demonstrated that FH is a broader spectrum marker of atherosclerosis-associated diseases. Due to its well-defined vascular injury, FH can be a potential target for TIA warning and for the early treatment of ischemic stroke.

Here, we have analyzed the biological function of ALDOA and FH. Our goal was to explain the physiological mechanism behind the elevation of related antibody levels in TIA patients. Based on our results, we inferred that the elevated levels of ALDOA and FH antibody levels represent risk predictors of ischemic stroke. Next, we aimed at demonstrating that the elevated levels of ALDOA-Abs and FH-Abs were independent risk factors for TIA and ischemic stroke. To this end, 92 TIA patients and 285 HDs were pooled together to establish a logistic regression analysis model ($n = 377$). It is a well-known fact that there are numerous independent risk factors affecting the occurrence and development of atherosclerosis, other than age, smoking habits, DM, HT, obesity and CHDs [7, 26–29, 53]. In the present model, we evaluated the association between TIA risk factors and/or antibody markers with the occurrence of TIA events (Table 3). The results showed that age, HT, DM, hyperlipidemia, and CHD had a good correlation with this model. It is indicated that this model is satisfied to analyze whether the elevated levels of ALDOA-Abs and FH-Abs are independent risk factors for TIA. By univariate and multivariate logistic regression analysis, we demonstrated that ALDOA-Abs and FH-Abs were diagnostic markers of TIA (Table 3). Since TIA is one of prodromal stages of CI, ALDOA-Abs and FH-Abs may be used as risk predictors. Specifically, they may have a high predictive value for ischemic stroke. To test this hypothesis, we conducted a case-control study nested within the Japan Public Health Center-based Prospective Study (see methods for details). ALDOA and FH's antibody levels were measured in 202 cases of incident cerebral infarction developed in patients of the cohort between the baseline and 2008, and in 202 controls with matching age, sex,

and area. In order to estimate the levels of ALDOA and FH for CI, we used a conditional logistic regression model. Our results showed that ALDOA and FH's antibody levels were positively and strongly associated with the risk of cerebral infarction. As a consequence, we believe that such antibody markers can be applied to predictive diagnosis rather than simple risk evaluation.

ALDOA-Abs and FH-Abs represent promising biomarkers for TIA and CI. Positive rates of each marker may not be sufficiently high. This may be due to their association with different causes (e.g., hypoxemia, HT, and smoking habit). In our opinion, the diagnostic value will improve through a combination of the measurement of antibodies and clinical risk factors, including age, HT, DM, and hyperlipidemia, which were independent TIA predictive factors in the multivariate logistic regression analysis (Table 3). Take TIA as an example, we calculated in the cohort of 92 patients with TIA and 285 HDs the positive rates including the conventional risk factors, age, HT, and DM. We used the cutoff values of ALDOA-Abs and FH-Abs to detect TIA, 14,869 and 2,849, respectively, as mentioned above. Positive predictive values (PPVs) of age, HT, and DM alone were 48.0%, 51.3%, and 71.1%, respectively (Supplementary Table 1). On the contrary, PPVs of ALDOA-Abs combined with age, HT, and DM increased to 63.1%, 63.5%, and 91.3%, respectively. Similarly, PPVs of FH-Abs combined with age, HT, and DM were 61.9%, 56.9%, and 94.1%, respectively. Furthermore, PPVs with the combination of three factors, HT, DM, and ALDOA-Abs or age, DM, and FH-Abs, reached up to 100% (Supplementary Table 1). In fact, antibody levels combined with clinical risk factors improve the ability to predict TIA, and this same applies to early prediction CI. Additional studies are needed to establish the complete predictive diagnosis system of ischemic stroke.

Collectively, this study provided solid evidence that ALDOA-Abs and FH-Abs could be used to early predict TIA and CI. ALDOA-Abs and FH-Abs' expression levels were independent warning markers for TIA and CI, providing additional information to guide therapeutic strategies.

Conclusions

The antibody levels against ALDOA and FH were significantly higher in patients with atherothrombotic TIA or CI than in HDs. As a consequence, they could be used as potential biomarkers for TIA and CI for an early prediction of the onset of ischemic stroke.

Declarations

Ethics approval and consent to participate

This study was reviewed and approved by both the Local Ethical Review Board of the Graduate School of Medicine, Chiba University, and by the Ethical Review Board of co-operating universities, hospitals, and research institutes. Informed written consent was obtained from all participants for all the studies.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

This work was performed in collaboration with Fujikura Kasei Co., Ltd. GT, RN, NSh, and HK are employees of Fujikura Kasei Co., Ltd.

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Author contributions

HW, EK, YI, KG, AX and TH conceived and designed the experiments. HW, XMZ, YY, KS, GT and RN performed the experiments. HW, HL, HI, MS, KY, NSa, ST, AAd, TMat and TMac analyzed and interpreted the data. SM, IK, TW, AAo, HT, KK, HS, NSh and HK contributed reagents, materials, analysis tools or data. HW, HL, EK, YI, KY, NSa, AX and TH wrote the paper.

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Figures

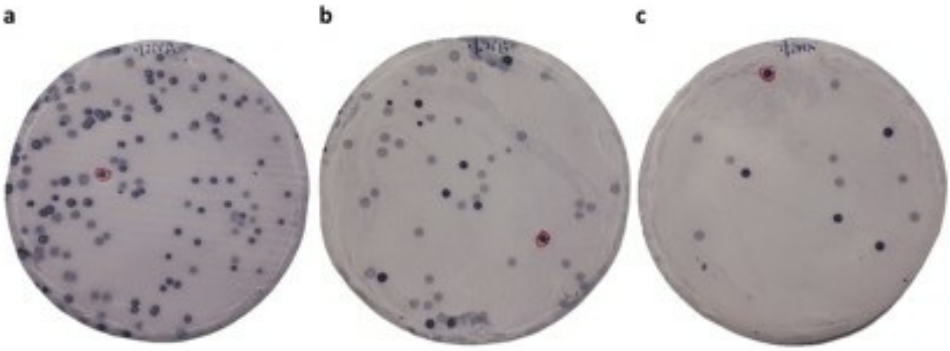

Figure 1

Immunoscreening of TIA antigens by SEREX. Recombinant expression cloning proteins were blotted on nitrocellulose membranes and reacted with sera originating from 19 TIA patients. Arrows indicate positive phage clones. Positive clones were re-cloned twice to obtain monoclonality.

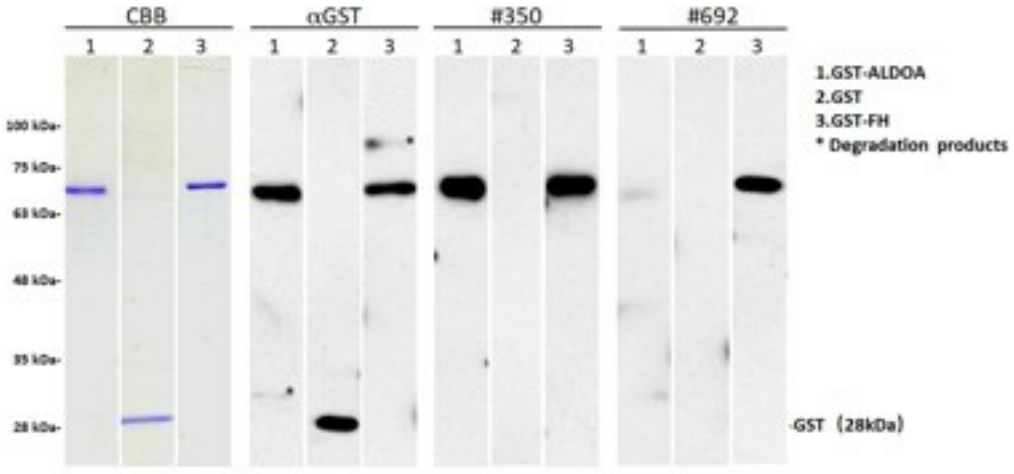

Figure 2

The presence of serum antibodies against ALDOA and FH antigenic proteins. Representative results of Western blotting are shown. The latter showed the detection at the expected sized of all the affinity purified GST fusion antigenic proteins (GST-ALDOA: 65kDa and GST-FH: 67kDa). GST and GST fusion proteins were electrophoresed through SDS-polyacrylamide gels. They were subsequently stained with Coomassie Brilliant Blue or Western blotting using anti-GST (αGST) or patient sera (#350 and #692). The specific reactions to GST-ALDOA and GST-FH are shown. The star represents degradation products post-electrophoresis. On the left of the figure are shown molecular weights.

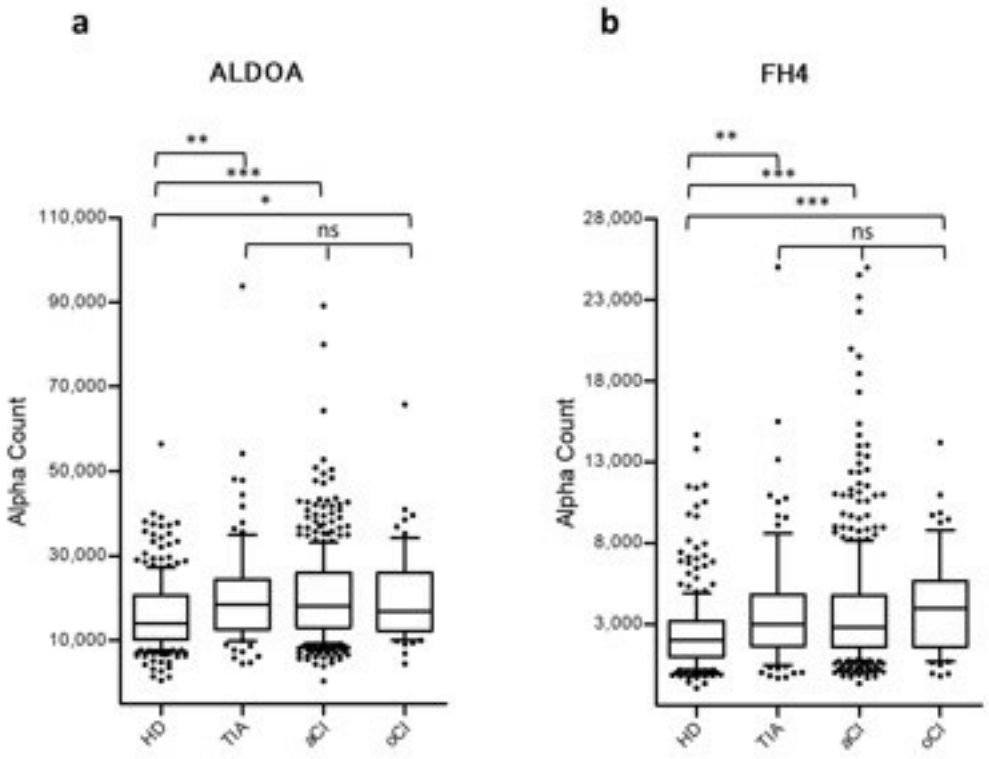

Figure 3

Comparison of serum ALDOA-Abs and FH-Abs levels between HDs and TIA, aCI or oCI patients. Antigens used were GST-ALDOA (a), and GST-FH (b). Following subtraction of the levels against control GST, serum levels of antibodies were examined by AlphaLISA are shown using a box-whisker. P values vs. HD specimens are shown as the stars. One star indicates $P < 0.05$, two stars indicate $P < 0.01$, three stars indicate $P < 0.001$. Table 2 shows the averages, SDs, total numbers, and P values.

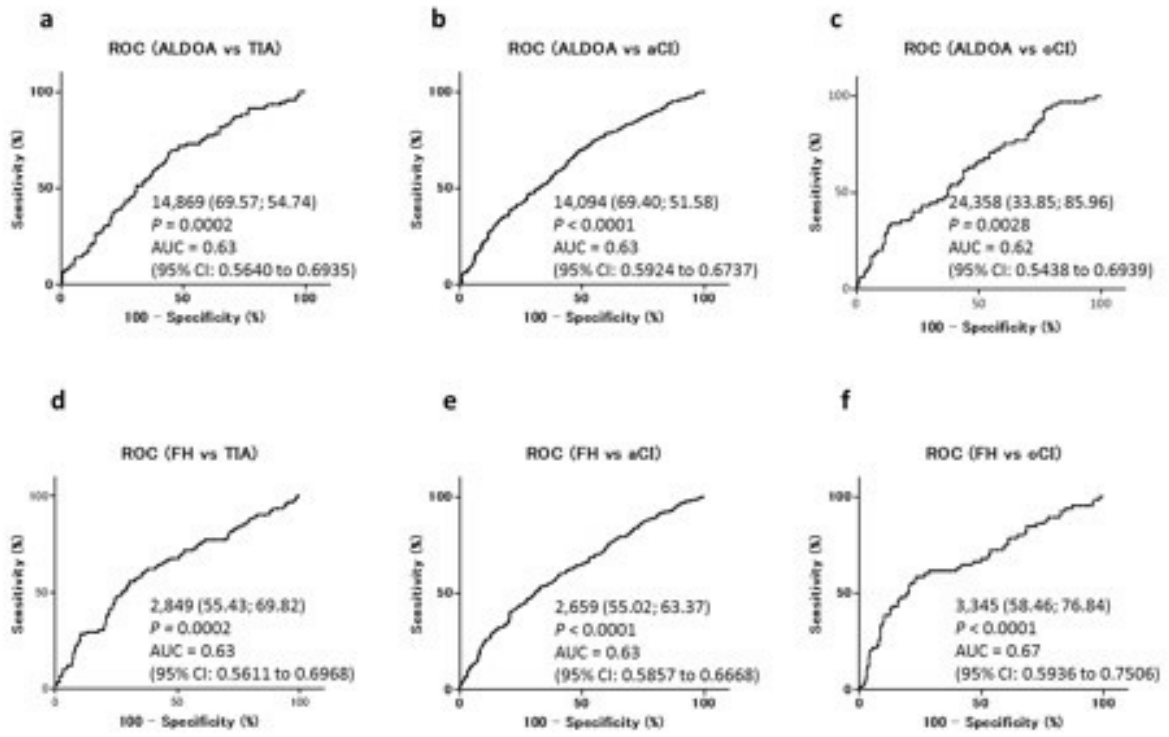

Figure 4

ROC analysis of ALDOA-Abs and FH-Abs for the prediction of TIA, aCI or oCI. Numbers in the figures indicate cutoff values for marker levels. Numbers in parentheses indicate sensitivity (left) and specificity (right). Areas under the curve (AUC), 95% confidence intervals (CI), and P values are shown.

Supplementary Files

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Supplementary Table_20200110.docx