Identification and Clinical Associations of 3 Forms of Circulating T-cadherin in Human Serum

Shiro Fukuda,1,2 Shunbun Kita,1,2 Kazuya Miyashita,3 Masahito Iioka,1 Jun Murai,4 Tadashi Nakamura,4 Hitoshi Nishizawa,1 Yuya Fujishima,1 Jun Morinaga,5 Yuichi Oike,5 Norikazu Maeda,1,6 and Iichiro Shimomura1

1Department of Metabolic Medicine, Graduate School of Medicine, Osaka University, Osaka, 565-0871, Japan; 2Department of Adipose Management, Graduate School of Medicine, Osaka University, Osaka, 565-0871, Japan; 3Immuno-Biological Laboratories Co., Ltd., Gunma, 375-0005, Japan; 4Department of Diabetes and Endocrinology, Kawasaki Hospital, Kobe, 652-0042, Japan; 5Department of Molecular Genetics, Graduate School of Medical Sciences, Kumamoto University, Kumamoto, Japan; and 6Department of Metabolism and Atherosclerosis, Graduate School of Medicine, Osaka University, Osaka, 565-0871, Japan

ORCID numbers: 0000-0003-0712-7154 (S. Fukuda); 0000-0002-8937-0053 (S. Kita).

Abbreviations: ALT, alanine transaminase; APN, adiponectin; AST, aspartate transaminase; BMI, body mass index; BNP, brain natriuretic peptide; CCA, common carotid artery; DBP, diastolic blood pressure; eGFR, estimated glomerular filtration rate; ELISA, enzyme-linked immunosorbent assay; eVFA, estimated visceral fat area; GPI, glycosylphosphatidylinositol; HbA1c, hemoglobin A1c; HDL-C, high-density lipoprotein cholesterol; HRP, horseradish peroxidase; hs-CRP, highsensitivity C-reactive protein; IMT, intima-media thickness; LDL-C, low-density lipoprotein cholesterol; MCV, motor nerve conduction velocity; MSC, mesenchymal stem/stromal cell; PWV, pulse wave velocity; SBP, systolic blood pressure; SDS, sodium dodecyl sulfate; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; SNP, single-nucleotide polymorphism; T-cad, T-cadherin; TG, triglyceride; UA, uric acid; WC, waist circumference; WT, wild type.

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Abstract

Context: T-cadherin (T-cad) is a glycosylphosphatidylinositol (GPI)-anchored cadherin that mediates adiponectin to induce exosome biogenesis and secretion, protect cardiovascular tissues, promote muscle regeneration, and stimulate therapeutic heart protection by transplanted mesenchymal stem cells. CDH13, the gene locus of T-cad, affects plasma adiponectin levels most strongly, in addition to affecting cardiovascular disease risk and glucose homeostasis. Recently, it has been suggested that T-cad exists in human serum, although the details are still unclear.

Objective: To validate the existence of T-cad forms in human serum and investigate the association with clinical parameters of type 2 diabetes patients.

Methods: Using newly developed monoclonal antibodies against T-cad, pooled human serum was analyzed, and novel T-cad enzyme-linked immunosorbent assays (ELISAs)
Adiponectin (APN) is an adipocyte-derived circulating factor that protects organs and tissues. The plasma APN concentration is inversely correlated with body weight and body mass index (BMI) (1, 2). Clinical association studies have suggested that hypoadiponectinemia is a risk factor for diabetes, cardiovascular diseases, and other diseases (3-7). Although single-nucleotide polymorphisms (SNPs) near or in the ADIPOQ locus, which affect the plasma APN level, had no or little impact on insulin sensitivity, diabetes, and coronary heart disease risks in Mendelian randomization studies (8, 9), SNPs outside the ADIPOQ locus were significantly associated with coronary heart disease risk (9). Their gene locus in a genome-wide association study was CDH13 (T-cadherin [T-cad] gene) (10). It causally affected both coronary artery disease and glucose homeostasis in a genome-wide association study (10, 11).

T-cad is classified as a classical cadherin with 5 extra-cellular cadherin repeats, but it is a unique cadherin with a glycosylphosphatidylinositol (GPI) anchor instead of transmembrane and intracellular domains. T-cad is mainly expressed in vascular endothelial and heart and skeletal muscle cells and binds multimeric APN, including hexamers and larger multimers (12-14). We and others also demonstrated that the cardiovascular protective and muscle regenerative effects of APN depended on T-cad in mice (15-19). Native APN in serum binds to cells expressing T-cad but not to those expressing other proposed binding partners, such as AdipoR or Calreticulin, with comparable stoichiometry (14). Native APN stimulated exosome biogenesis and secretion by binding to T-cad (17). Recently, we found that mesenchymal stem/stromal cells (MSCs) expressed T-cad and produced many exosomes in response to APN (20). Heart protection by transplanted MSCs in a heart failure mouse model requires circulating APN and T-cad expression in MSCs to produce exosomes (20).

Unlike other cadherins, a proportion of mature T-cad uniquely retains an unprocessed prodomain at the N-terminus, even after being expressed on the cell surface (21). By analyzing the domain structure of T-cad, we revealed the importance of this prodomain for APN binding. Namely, the 130-kDa T-cad has a prodomain localized on the cell surface and binds more APN than the 100-kDa T-cad without the prodomain (13).

We and others identified T-cad in plasma associated with extracellular vesicles. Following this observation, Philippova and colleagues developed an enzyme-linked immunosorbent assay (ELISA) using commercially available antibodies and reported that the plasma T-cad concentration is associated with the incidence of acute coronary syndrome and high-molecular-weight APN levels (22, 23). Furthermore, T-cad in plasma was detected by a commercially available ELISA (24) and mass spectrometry-based proteomics analysis (25), suggesting that plasma T-cad is a potential biomarker. However, these analyses were unable to discriminate between the 130-kDa full-length form and 100-kDa form without the prodomain. Whether all T-cad exists as the cargo of extracellular vesicles has not been clarified. We thus developed a new series of monoclonal antibodies against human T-cad; confirmed the presence of vesicle-free forms of the 130-kDa full-length form, 100-kDa form without the prodomain, and 30-kDa prodomain in plasma; and developed ELISAs that can discriminate these forms. We also found that the 3 forms of soluble T-cad were differentially associated with clinical parameters, including plasma APN levels, in diabetes patients.

Materials and Methods

Animals and cells

A total of 6 Wistar rats (6 weeks old, male; Charles River) and 3 Cdh13<del>del</del> mice (13 weeks old, male) were used to raise antibodies against human T-cad. Cdh13<del>del</del> (systemic...
T-cad knockout) mice were established by crossbreeding Cdh13^flox/flox mice (a gift from Dr. Christopher Hug, Boston Children’s Hospital) with E2a-Cre mice (Stock no: 003724; The Jackson Laboratory). Human adipose-derived mesenchymal stem cells were purchased (#PT-5006; Lonza). This study was approved by the Ethics Review Committee for Animal Experimentation of Osaka University School of Medicine and conducted in accordance with the Guide for the Care and Use of Laboratory Animals published by the United States National Institute of Health.

Immunoprecipitation and Western Blotting

A standard immunoprecipitation procedure was used to verify the novel anti-T-cad monoclonal antibodies. Briefly, each antibody was biotinylated using an Ez-Link Sulfo-NHS-LC-Biotin kit (#21435; Thermo) according to the manufacturer’s instructions. One microgram of the biotinylated antibody and 100 µg of human adipose-derived mesenchymal stem cell lysate solubilized with 20 mM Tris (pH 7.4), 150 mM NaCl, 5 mM EDTA, 1% NP-40, and cOmplete Protease Inhibitor Cocktail (#11873580001; Roche) were mixed and pulled down by streptavidin sepharose (#17511301; Cytiva). Following sufficient washing, the bound fraction was eluted by adding Laemmli sodium dodecyl sulfate (SDS) sample buffer with heating at 100°C for 5 minutes. The eluted samples were analyzed by SDS-polyacrylamide gel electrophoresis and immunoblotting. For the immunoblotting of T-cad, goat polyclonal antihuman cadherin13 antibody (#AF3264; R&D), which was manually biotinylated using an Ez-Link Sulfo-NHS-LC-Biotin kit (same as above), was used as the primary antibody at 0.5 µg/mL, and horseradish peroxidase (HRP)-conjugated streptavidin (#N100; Thermo) was used as the secondary antibody at 0.125 µg/mL. To detect exosome-specific proteins, anti-Tsg101 (host: rabbit, ab125011, Abcam), and anti-Syntenin (host: rabbit, ab133267, Abcam) antibodies were used at 0.5 µg/mL, and antirabbit immunoglobulin G and HRP-linked whole-Ab donkey (NA934, Cytiva) were used at a 5000-fold dilution.

Purification of Soluble T-cad from Human Serum

Two or 3 mg of anti-T-cad antibody m11 or h6 was directly immobilized on a 1-mL HiTrap NHS-activated HP column (#17071601; Cytiva) according to the manufacturer’s instructions. The antibody(−) column (nothing immobilized) was used as a negative control. After equilibration with binding buffer (20 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1% NP-40, and 5 mM EDTA), 200 mL of pooled human serum (#12181201, lot no. BJ13091A; CosmoBio, Japan) with 20 mM Tris-HCl (pH 7.4), 1% NP-40, and 5 mM EDTA was applied to each column at a flow rate of 0.5 mL/minute. Following sufficient washing (approximately 10 column volumes) with binding buffer, the bound fraction from each column was eluted with 5 mL of 3 M MgCl₂ at a flow rate of 0.25 mL/minute. The bound fraction was desalted using a PD-10 column (#17085101; Cytiva) and concentrated with Amicon Ultra-0.5 3k devices (#UFC500324; Merck).

Identification of Human Soluble T-cad

Purified soluble T-cad from human serum was subjected to SDS-PAGE and visualized by silver staining using EzStain Silver (#AE-1360; Atto, Japan) according to the manufacturer’s instructions. For nano-liquid chromatography/mass spectrometry, significantly different bands between m11-purified and h6-purified soluble T-cad (bands (1)-(4) in Fig. 1E and 1F) were analyzed using an UltiMate 3000 Nano LC system, Q-Exactive (Thermo) at CoMIT Omics Center of Osaka University. The obtained MS data were analyzed using Scaffold software version 4.4.3 (Proteome Software Inc.).

Gel Filtration Analysis

One milliliter of the pooled human serum (see “Purification of soluble T-cad from human serum”) was separated on a HiLoad 16/60 Superdex 200 prep grade gel-filtration column (#17106901; Cytiva) equipped on an AKTA pure 25 M1 (Cytiva) in buffer containing 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 1 mM CaCl₂. The flow rate was 1.0 mL/minute, and the room temperature was 25°C. Each 1-mL fraction was collected and analyzed. A Gel Filtration Calibration Kit HMW (#28403842; Cytiva) was used as a molecular weight standard.

Construction and Validation of Novel T-cad ELISA

The construction strategy for the novel T-cad ELISA is shown in Fig. 2A. The coefficients of variation (%CVs) were determined at 3 different concentrations. The intra-assay %CVs were 4.4% to 7.1% for 130 kDa + 100 kDa, 9.0% to 9.6% for 130 kDa, and 6.4% to 7.5% for 130 kDa + 30 kDa. The interassay %CVs were 5.0% to 12.1% for 130 kDa + 100 kDa, 5.3% to 7.4% for 130 kDa, and 6.4% to 7.5% for 130 kDa + 30 kDa. The minimum detectable concentrations were 0.36 pmol/L for 130 kDa + 100 kDa, 0.24 pmol/L for 130 kDa, and 0.18 pmol/L for 130 kDa + 30 kDa. These ELISA kits are not commercially available currently, but they will be launched in the near future (see Disclosures).
Exosome isolation

Exosome isolation from human serum was performed as reported previously (17), with some modifications. Briefly, pooled human serum (#12181201, lot No. BJ14005A; CosmoBio, Japan) was passed through a 0.45-μm PVDF filter (#SLHV033RB, Merck) to remove crude aggregates. Approximately 40 mL of filtered serum was ultracentrifuged at 110 000 g for 2 hours, followed by washing the exosome pellet with 20 mM Tris (pH 7.4), 150 mM NaCl, and 2 mM CaCl₂ at 110 000 g for 2 hours (rotor: SW32Ti/ultracentrifuge: L-90K, Beckman Coulter). The exosome pellets were solubilized in 200 μL of 20 mM Tris (pH 7.4), 150 mM NaCl, 5 mM EDTA, 1% NP-40, and 0.02% NaN₃. The exosome sample was analyzed by Western blotting and subjected to the new T-cadherin ELISA at an approximately 100-fold dilution.

Subjects

The study subjects were 183 patients with type 2 diabetes mellitus who were admitted to the Division of Diabetes and Endocrinology, Kawasaki Hospital, Kobe, Japan,
Figure 2. New T-cadherin enzyme-linked immunosorbent assays (ELISAs) were developed to identify the 3 forms of T-cadherin. (A) The strategy of newly developed T-cadherin (T-cad) ELISAs. The combination of cadherin repeat-recognizing antibodies (m11 and K59 in Fig. 1A) detects the 130-kDa and 100-kDa forms of T-cad, which have an extracellular domain (upper). Similarly, the combination of cadherin repeat-recognizing and prodomain-recognizing antibodies (m11 and h6) detects the 130-kDa form of T-cad, which has the prodomain and extracellular domain (middle), and the combination of prodomain-recognizing antibodies (h5 and h6) detects the 130-kDa and 30-kDa forms of T-cad, which have the prodomain (bottom). The concentrations of the 100-kDa and 30-kDa T-cad were calculated as (130 kDa + 100 kDa) – (130 kDa), (130 kDa + 30 kDa) – (130 kDa), respectively. The recognized domain and host of antibodies used in these ELISAs are also shown. (B-E) Consideration of several conditions for measuring soluble T-cadherin (T-cad) in human serum or plasma using the ELISAs shown in A (triplicate data, mean ± SE). (B) Serum or plasma (EDTA). (C) Serum repeatedly freeze-thaw. (D) Preprandial or postprandial serum. (E) Overnight stability at 37°C. In B, the measured values (130 kDa + 100 kDa, 130 kDa, and 130 kDa + 30 kDa) and the subtracted values (100 kDa and 30 kDa) are shown to clarify the strategy of the ELISA. Student's t-test or Dunnett's test was performed, and the probability value (P) is shown.
improve their glycemic control and/or receive treatment for complications between August 2015 and April 2017, as reported previously (26). Each subject provided written informed consent after the purpose and possible complications of the study were explained. T-cad concentrations were measured using remaining serum from the study by the newly established T-cad ELISA described above. This study was approved by the Human Ethics Committees of Osaka University (no. 15061).

Statistical Analysis

Data are presented as the mean ± SD or median (interquartile range). For variables with a skewed distribution, logarithmic transformation was performed for correlation analysis. P < .05 was considered statistically significant. The Bonferroni correction was used if needed. All analyses were carried out using JMP Pro 13.2.1 for Windows (SAS Institute).

Results

The Existence of 3 Forms of T-cad in Human Serum

We developed monoclonal antibodies against recombinant human T-cad in immunized WT rats or T-cad null mice. Through rounds of screening, 4 clones were obtained that recognized different domains of human T-cad (Fig. 1A). Western blots of each immunoprecipitate of human mesenchymal stem cell lysate, which expresses human T-cad (20), using the respective monoclonal antibodies are shown in Fig. 1B. Clones m11 and K59 precipitated the 130-kDa T-cad, demonstrating that these clones recognized cadherin repeats 1 through 5. The other 2 clones, h5 and h6, precipitated only 130-kDa T-cad, suggesting that they recognized the prodomain (Fig. 1B).

Next, we tried to isolate soluble T-cad in human serum (Fig. 1C). After applying human serum to m11- or
h6-immobilized columns and washing, bound proteins were subjected to SDS-PAGE. Compared with the band pattern of nonspecifically associated proteins from the antibody-minus (Ab(−)) column with Coomassie staining (Fig. 1D) and subsequent Western blotting by an anti-T-cad polyclonal antibody (Fig. 1E), m11-coupled resin produced visible bands for the 130-kDa full-length and 100-kDa T-cad (bands 1 and 3 in Fig. 1D and 1E), and clone h6 produced bands for the 130-kDa T-cad (bands 2 in Fig. 1D and 1E). In addition, a 30-kDa band was detected after a longer exposure (band 4 in Fig. 1E). To identify these proteins, the corresponding bands in a silver-stained gel were subjected to mass spectrometry analysis (Fig. 1F, G). From band 1 or 2, corresponding to the 130-kDa T-cad, peptides spanning the prodomain and cadherin repeats were detected (Fig. 1D-G). From band 3, corresponding to the 100-kDa T-cad, peptides were mapped to cadherin repeats but not to the prodomain (Fig. 1D-1G). From band 4, only peptides mapped on the prodomain were detected (Fig. 1E-1G). Collectively, our monoclonal antibodies and mass spectrometry spec–based analysis identified the 130-kDa full-length, 100-kDa, and 30-kDa prodomain T-cad in serum.

Construction of Novel T-cad ELISA

Next, we created 3 different ELISAs by combining monoclonal antibodies confirmed to recognize the forms of native human soluble T-cad (Fig. 2A). The first evaluated T-cad with cadherin repeats (130 kDa + 100 kDa), the second directly measured T-cad with both cadherin repeats and the prodomain (130 kDa), and the third evaluated T-cad with the prodomain (130 kDa + 30 kDa). From these values, the 100-kDa T-cad and 30-kDa prodomain were calculated by subtraction from the 130-kDa form. The concentrations of soluble T-cad molecular species were not significantly different between plasma and serum after repeated freezing and thawing, after a meal, or after overnight incubation at 37°C (Fig. 2B-2E).

The Presence of Monomeric Soluble T-cad in Human Serum

Using this set of ELISAs, we analyzed soluble T-cad forms in human serum by gel filtration. The gel filtration profile of human serum T-cad and APN using the HiLoad 16/60 Superdex 200 prep grade column is shown in Fig. 3A. The 130-kDa T-cad appeared first, followed by the 100-kDa and 30-kDa forms. Undetectable levels of T-cad were observed at the size exclusion limit, corresponding to extracellular vesicle-associated T-cad. As we demonstrated the presence of T-cad on exosomes in a previous report (17), we examined whether exosome-associated T-cad was measurable by the new ELISAs. Using an ultracentrifugation method, exosomes were isolated from human serum (Fig. 3B, left). T-cad on human serum exosomes was detected by Western blotting (Fig. 3B, right) and by the new T-cad ELISAs (Fig. 3C). Exosome-associated T-cad was mainly

| Table 1. Clinical characteristics of the study subjects |
|-----------------------------------------------|
| Variables                                      |
| n (males/females)                             | 183 (126/57) |
| Age (years)                                   | 64.7 ± 12.6  |
| Duration of diabetes (years)                  | 9 (3–21)     |
| BMI (kg/m²)                                   | 25.3 ± 4.7   |
| WC (cm)                                       | 93.8 ± 12.9  |
| eVFA (cm²)                                    | 137.7 ± 57.8 |
| Grip strength (kg)                            | 23.5 ± 9.5   |
| Skeletal mass index (kg/m²)                   | 7.1 ± 1.2    |
| SBP (mmHg)                                    | 131.7 ± 19.3 |
| DBP (mmHg)                                    | 76.4 ± 12.0  |
| HbA1c (%)                                     | 9.5 ± 2.1    |
| Serum C-peptide (ng/mL)                       | 1.8 ± 1.3    |
| AST (U/L)                                     | 22 (17–30)   |
| ALT (U/L)                                     | 21 (14–35)   |
| UA (mg/dL)                                    | 5.3 ± 1.6    |
| cGFR (mL/min)                                 | 67.3 ± 28.3  |
| HDL-C (mg/dL)                                 | 46.1 ± 16.8  |
| TG (mg/dL)                                    | 148 (105–217)|
| LDL-C (mg/dL)                                 | 106.5 ± 36.8 |
| hs-CRP (mg/dL)                                | 0.12 (0.06–0.35) |
| BNP (pg/mL)                                   | 15.7 (7.7–35.3) |
| CCA max IMT (mm)                              | 0.9 ± 0.5    |
| CCA mean IMT (mm)                             | 0.8 ± 0.3    |
| PWV (m/sec)                                   | 1810 ± 379   |
| MCV (m/sec)                                   | 49.5 ± 4.4   |
| Amplitude of the median nerve (µV)            | 7.5 ± 2.2    |
| Hypertension                                  | 68%          |
| Hyperuricemia                                 | 26%          |
| Dyslipidemia                                  | 86%          |
| Cardiovascular disease                        | 34%          |
| Fatty liver                                   | 58%          |
| Adiponectin (µg/mL)                           | 7.3 (4.6–11.3) |
| 130-kDa T-cadherin (pmol eq./L)               | 558.6 ± 189.2 |
| 100-kDa T-cadherin (pmol eq./L)               | 1136.2 ± 419.2 |
| 30-kDa T-cadherin (pmol eq./L)                | 1182.9 ± 685.9 |

Continuous variables are shown as the mean ± SD or median (lower-higher quartile). The concentration of the 130-kDa/100-kDa/30-kDa T-cadherin is represented by the recombinant 130-kDa T-cadherin equivalent. Rows from “n (males/females)” to “Adiponectin (µg/mL)” were replicated from our previous report: Murai et al. [26].

Abbreviations: BMI, body mass index; WC, waist circumference; eVFA, estimated visceral fat area; SBP, systolic blood pressure; DBP, diastolic blood pressure; HbA1c, hemoglobin A1c; AST, aspartate transaminase; ALT, alanine transaminase; UA, uric acid; cGFR, estimated glomerular filtration rate; HDL-C, high-density lipoprotein cholesterol; TG, triglyceride; LDL-C, low-density lipoprotein cholesterol; hs-CRP, high-sensitivity C-reactive protein; BNP, brain natriuretic peptide; CCA, common carotid artery; IMT, intima-media thickness; PWV, pulse wave velocity; MCV, motor nerve conduction velocity.
composed of the 130-kDa form, with a different distribution from serum T-cad forms. The amount of T-cad in the exosome fraction was much smaller than that in the soluble fraction of human serum. Each soluble T-cad form was eluted in fractions corresponding to their respective molecular mass in gel filtration analysis (Fig. 3A), suggesting that each form exists as a monomer. Although hexameric and multimeric high-molecular-weight APN are known to bind membrane-anchored T-cad with strong affinity, soluble T-cad was eluted independent of APN.

Correlations between Soluble T-cad and Clinical Characteristics in Japanese Type 2 Diabetes Patients

We explored the distribution of soluble T-cad concentrations in Japanese type 2 diabetes patients. This was an additional analysis that we previously reported for the other clinical investigation (26). All 183 subjects were included in this analysis. The concentrations of the 130-kDa, 100-kDa, and 30-kDa T-cad forms in each remaining serum sample were measured by the newly developed ELISA. The clinical characteristics and T-cad concentrations of the subjects are shown in Table 1.

First, we examined the correlations among forms of soluble T-cad (Fig. 4). All T-cad forms, including the 100-kDa and 30-kDa forms, which were calculated by subtraction of (130 kDa + 100 kDa) – (130 kDa) and (130 kDa + 30 kDa) – (130 kDa), were present at detectable concentrations. The average concentration of each soluble T-cad (recombinant 130-kDa T-cad equivalent) was 558.6 ± 189.2 pmol/L for the 130-kDa T-cad, 1136.2 ± 419.2 pmol/L for the 100-kDa T-cad, and 1182.9 ± 685.9 pmol/L for the 30-kDa T-cad. The levels of soluble T-cad were approximately 100-fold less than the levels of serum APN (4.6-11.3 μg/mL, equal to 51.1-125.6 nmol/L of the trimer equivalent) (Table 1). There was a significant positive correlation (r = 0.47, P < .001) between the 130-kDa and 100-kDa T-cad concentrations (Fig. 4A). However, the 30-kDa T-cad had no significant correlation with the 100-kDa (Fig. 4B) or 130-kDa T-cad (Fig. 4C).

Next, we assessed the correlations between soluble T-cad and APN, the binding partner of membrane-associated T-cad (12-14). Both the 130-kDa (Fig. 4D) and 30-kDa (Fig. 4F) T-cad exhibited significant positive correlations (130 kDa; r = 0.30, P < .001, 30 kDa; r = 0.23, P < .01) with APN, whereas no significant correlation was observed between the 100-kDa T-cad (Fig. 4E) and APN.

Finally, we performed simple linear regression analyses between serum soluble T-cad and clinical parameters (Tables 2-4). Both the unadjusted model and the age-, sex-, and BMI-adjusted models were applied. The serum concentration of the 130-kDa T-cad was significantly correlated with BMI, waist circumference (WC), estimated visceral fat area
(eVFA), high-density lipoprotein cholesterol (HDL-C), triglyceride (TG), high-sensitivity C-reactive protein (hs-CRP), brain natriuretic peptide (BNP), and APN in the unadjusted model (Table 2). After adjusting for age, sex, and BMI, the 130-kDa T-cad was significantly correlated with HDL-C, hs-CRP, and APN (Table 2). Regarding the 100-kDa T-cad, a significant correlation was observed only with eGFR, even in the adjusted model, and weak correlations with HbA1c and serum C-peptide were noted (Table 3). On the other hand, the serum concentration of the 30-kDa T-cad was significantly correlated with several clinical parameters in the unadjusted model (Table 4). Even after adjusting for age, sex, and BMI, the 30-kDa T-cad was significantly correlated with HbA1c, serum C-peptide, ALT, uric acid (UA), eGFR, BNP, motor neuron conduction velocity (MCV), and the amplitude of the median nerve (Table 4).

### Table 2. Correlations between the serum concentration of the 130-kDa T-cadherin and clinical parameters

| Variables                        | Unadjusted           | Age-, sex-, and BMI-adjusted |
|----------------------------------|----------------------|------------------------------|
|                                  | n        | r         | P      | std β  | P      |
| Age                              | 183      | 0.089     | .231  |       |        |
| log(Duration of diabetes)        | 153      | 0.123     | .159  | 0.090 | .304  |
| BMI                              | 183      | -0.238    | .001  |       |        |
| WC                               | 183      | -0.230    | .002  | -0.132| .411  |
| eVFA                             | 183      | -0.197    | .008  | -0.227| .128  |
| Grip strength                    | 183      | 0.126     | .088  | 0.169 | .112  |
| Skeletal mass index              | 183      | 0.000     | .997  | 0.245 | .091  |
| Serum C-peptide                  | 177      | 0.125     | .097  | 0.130 | .797  |
| log(AST)                         | 183      | 0.083     | .265  | 0.131 | .799  |
| log(ALT)                         | 183      | -0.010    | .897  | 0.076 | .324  |
| UA                               | 183      | -0.060    | .419  | -0.047| .536  |
| eGFR                             | 183      | -0.097    | .192  | -0.148| .066  |
| HDL-C                            | 183      | 0.259     | <.001 | 0.259 | <.001 |
| log(TG)                          | 183      | -0.167    | .024  | -0.108| .181  |
| LDL-C                            | 183      | 0.061     | .409  | 0.090 | .231  |
| log(hs-CRP)                      | 173      | -0.315    | <.001 | -0.263| <.001 |
| log(BNP)                         | 179      | 0.191     | .010  | 0.161 | .081  |
| CCA max IMT                      | 162      | -0.009    | .913  | -0.058| .463  |
| CCA mean IMT                     | 168      | 0.016     | .833  | -0.044| .581  |
| PWV                              | 176      | 0.004     | .958  | -0.032| .720  |
| MCV                              | 172      | -0.090    | .240  | -0.074| .321  |
| Amplitude of the median nerve    | 174      | 0.103     | .176  | 0.097 | .217  |
| log(Adiponectin)                 | 183      | 0.284     | <.001 | 0.257 | .002  |

Logarithmic transformation was performed for variables with a skewed distribution. The number of analyzed subjects (n), Pearson’s correlation coefficient (r), standardized beta coefficients (std β), and each probability value (P) are shown. Values with P < .05 are presented in bold font. See Table 1 for abbreviations.

### Table 3. Correlations between the serum concentration of the 100-kDa T-cadherin and clinical parameters

| Variables                        | Unadjusted           | Age-, sex-, and BMI-adjusted |
|----------------------------------|----------------------|------------------------------|
|                                  | n        | r         | P      | std β  | P      |
| Age                              | 183      | -0.049    | .507  |       |        |
| log(duration of diabetes)        | 153      | -0.049    | .576  | -0.399| .669  |
| BMI                              | 183      | 0.073     | .328  |       |        |
| WC                               | 183      | 0.090     | .227  | 0.104 | .531  |
| eVFA                             | 183      | 0.088     | .234  | -0.003| .983  |
| Grip strength                    | 183      | 0.103     | .164  | 0.670 | .545  |
| Skeletal mass index              | 183      | 0.099     | .184  | -0.020| .896  |
| Serum C-peptide                  | 180      | 0.037     | .621  | 0.046 | .542  |
| HbA1c                            | 177      | 0.151     | .045  | 0.165 | .032  |
| log(AST)                         | 183      | 0.127     | .086  | 0.126 | .105  |
| log(ALT)                         | 183      | 0.092     | .216  | 0.066 | .411  |
| UA                               | 183      | 0.068     | .363  | 0.034 | .665  |
| eGFR                             | 183      | -0.186    | .012  | -0.247| .003  |
| HDL-C                            | 183      | -0.074    | .317  | -0.034| .670  |
| log(TG)                          | 183      | 0.132     | .076  | 0.126 | .132  |
| LDL-C                            | 183      | 0.055     | .462  | 0.040 | .609  |
| log(hs-CRP)                      | 173      | 0.082     | .286  | 0.088 | .261  |
| log(BNP)                         | 179      | -0.055    | .465  | 0.009 | .924  |
| CCA max IMT                      | 162      | 0.084     | .287  | 0.100 | .226  |
| CCA mean IMT                     | 168      | 0.070     | .365  | 0.091 | .275  |
| PWV                              | 176      | -0.084    | .265  | -0.076| .402  |
| MCV                              | 172      | -0.114    | .137  | -0.121| .119  |
| Amplitude of the median nerve    | 174      | -0.003    | .966  | -0.019| .803  |
| log(Adiponectin)                 | 183      | -0.129    | .082  | -0.118| .168  |

See the legend of Table 2 for more information and abbreviations.

**Discussion**

We demonstrated that (1) 3 forms of soluble T-cad, a 130-kDa full-length form, 100-kDa form, and 30-kDa prdomain, exist in human plasma. (2) The majority of soluble T-cad circulates as free protein independent from APN and extracellular vesicles. (3) The concentration of each soluble T-cad, especially the 30-kDa form, is associated with several clinical parameters in diabetes patients.

The existence of T-cad protein in blood was suggested by both lab-made and commercial ELISAs and mass spectrometry analysis of the plasma proteome (22, 23). However, their state in plasma had not been clarified. Although we and others reported that T-cad is associated with extracellular vesicles in plasma, membrane-anchored T-cad in plasma is the minority, and most T-cad circulates independent of vesicles, as described below. With gel-filtration chromatography, such vesicle-associated T-cad should elute at the beginning of the
Table 4. Correlations between the serum concentration of the 30-kDa T-cadherin and clinical parameters

| Variables                        | Unadjusted | Age-, sex-, and BMI-adjusted |
|----------------------------------|------------|-------------------------------|
|                                  | n  | r   | P     | stdβ | P     |
| Age (duration of diabetes)       | 183| 0.331| <.001|      |       |
| BMI                              | 183| −0.019| .800|      |       |
| WC                               | 183| −0.012| .871| −0.045| .774 |
| eVFA                             | 183| −0.017| .822| −0.113| .441 |
| Grip strength                    | 183| −0.201| .006| −0.196| .059 |
| Skeletal mass index              | 183| −0.012| .868| 0.268| .059 |
| SBP                              | 183| 0.184| .013| 0.154| .030 |
| DBP                              | 183| −0.074| .317| −0.007| .927 |
| HbA1c                            | 177| −0.327| <.001| −0.276| <.001 |
| Serum C-peptide                  | 180| 0.334| <.001| 0.364| <.001 |
| log(AST)                         | 183| −0.062| .402| −0.140| .055 |
| log(ALT)                         | 183| −0.186| .012| −0.207| .006 |
| UA                               | 183| 0.269| <.001| 0.308| <.001 |
| eGFR                             | 183| −0.650| <.001| −0.641| <.001 |
| HDL-C                            | 183| −0.081| .275| −0.110| .148 |
| log(TG)                          | 183| −0.016| .826| 0.140| .076 |
| LDL-C                            | 183| −0.252| <.001| −0.175| .016 |
| log(hs-CRP)                      | 173| 0.031| .683| 0.055| .455 |
| log(BNP)                         | 179| 0.391| <.001| 0.371| <.001 |
| CCA max IMT                      | 162| 0.248| <.001| 0.182| .018 |
| CCA mean IMT                     | 168| 0.282| <.001| 0.171| .029 |
| PWV                              | 176| 0.312| <.001| 0.181| .034 |
| MCV                              | 172| −0.397| <.001| −0.358| <.001 |
| Amplitude of median nerve        | 174| −0.236| .002| −0.204| .109 |
| log(Adiponectin)                 | 183| 0.210| .004| 0.122| .130 |

See the legend of Table 2 for more information and abbreviations.

The increased valency in a specific area of the plasma membrane and assistance by other plasma membrane constituents and/or inhibiting factors in serum may also have an influence.

The soluble form was also reported for other cadherins such as E-, N-, and P-cadherin in addition to T-cad (29). In contrast to these soluble classical cadherins that circulate in a single extracellular mature form, T-cad circulates in 3 different forms: the 130-kDa form with a prodomain, 100-kDa form without the prodomain, and 30-kDa prodomain. As T-cad can be sorted to the plasma membrane without prodomain processing, these different forms may be generated from the cell surface.

The plasma levels of soluble E-cadherin are correlated with a poor prognosis in ovarian cancer patients (29). Increases in soluble N- or P-cadherin (30) are also related to tumor progression (31). Soluble E-cadherin was reported to disrupt epithelial cell-cell junctions coordinated by transmembrane E-cadherin (32), which was recently reported to bind to the surface of extracellular vesicles and stimulate tumor angiogenesis (33). Similarly, soluble forms of T-cad may be important as clinical biomarkers and/or exert bioactivity.

Of note, the present study demonstrated that these 3 forms of soluble T-cad are differentially associated with clinical parameters in Japanese type 2 diabetes patients. The 130-kDa form is strongly associated with metabolically important factors, such as HDL-C, hs-CRP, and APN. We previously demonstrated that APN preferentially increased the 130-kDa T-cad in tissues and cells (13). It is possible that the levels of the soluble 130-kDa form in blood represent the tissue levels of GPI-anchored T-cad. The 100-kDa form, on the other hand, exhibited no significant association with clinical parameters except with eGFR and the 130-kDa form. The 30-kDa form was significantly associated with clinical parameters related to metabolic and cardiovascular disease, such as HbA1c, serum C-peptide, ALT, uric acid, LDL-C, intima-media thickness, and BNP. Most likely due to its small molecular mass, eGFR was strongly associated with the 30-kDa T-cad. Both MCV and the amplitude of the median nerve were also negatively associated with the 30-kDa form. The decrease in MCV and the amplitude of the median nerve are well reported in diabetic neuropathy (34). T-cad is highly expressed in neuronal cells in addition to muscle and endothelial cells (21). Thus, it is possible that the 30-kDa T-cad could be a marker for diabetic neuropathy, although further clinical investigations are needed. Many significant associations combined with the unique presence of cadherins suggest that these molecules may play important roles.
There are several important points that remain unclear. We revealed that at least 3 soluble forms of T-cad are present in human blood, but other forms may be present. Which T-cad-expressing organ these 3 forms are derived from is also unknown. Sources of abundant T-cad include the vascular endothelium, heart and skeletal muscle cells, central nerve tissue cells, and tissue-resident MSCs. How these 3 forms are produced and how their blood levels are controlled also remain unclear. Similar to other cadherins, T-cad may be cleaved from the cell membrane or extracellular vesicles. The enzyme involved in cleavage may be important. The normal range and clinical significance of the 3 forms of soluble T-cad should be clarified in larger prospective studies.

In conclusion, we demonstrated that 3 forms of soluble T-cad are present in human blood and are correlated with several clinical parameters in type 2 diabetes.

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Author Contributions: S.F. and S.K. designed the study protocol, performed the biochemical experiments, analyzed the data, and cowrote the manuscript. K.M. performed antibody screening and established and validated the new ELISAs. J.M. recruited the clinical study subjects and acquired data, and H.N. conceived the clinical study. S.F. and M.I. acquired and analyzed the additional data of the clinical study. Y.F., T.N., J.M., Y.O., N.M., and I.S. discussed and reviewed the manuscript. All authors read and approved the final manuscript.

Additional Information
Correspondence: Shunbun Kita, PhD, Osaka University, Suita, Osaka Japan. Email: shunkita@endmet.med.osaka-u.ac.jp.

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Data Availability: All raw data (except the clinical data in Fig. 4 and Tables 1-4) for this paper can be found at the following address: https://doi.org/10.5061/dryad.fqz612jr2. The datasets of the clinical data are not publicly available due to ethical restrictions but are available from the corresponding author upon reasonable request.

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