HLA-DQ genotype and biochemical characterization of anti-transglutaminase 2 antibodies in patients with type 1 diabetes mellitus in Taiwan

Yann-Jinn Lee1,2,3,4,5 | Wei-Hsin Ting2,3,6 | Yi-Wen Yang1 | Cheng-Jui Lin6,7 | Yu-Ting Hsieh1,8 | Chi-Yu Huang2,3,6 | Fu-Sung Lo9,10 | Chen-Chung Chu4 | Chiung-Ling Lin4 | Wen-Shan Lin4 | Thung-S. Lai1

1Institute of Biomedical Sciences, MacKay Medical College, New Taipei City, Taiwan, ROC
2Department of Medicine, MaKkay Medical College, New Taipei City, Taiwan, ROC
3Department of Pediatric Endocrinology, MacKay Children’s Hospital, Taipei, Taiwan, ROC
4Department of Medical Research, MacKay Memorial Hospital Tamsui District, New Taipei City, Taiwan, ROC
5Department of Pediatrics, School of Medicine, College of Medicine, Taipei Medical University, Taipei, Taiwan, ROC
6MacKay Junior College of Medicine, Nursing, and Management, Taipei, Taiwan, ROC
7Division of Nephrology, Department of Internal Medicine, Mackay Memorial Hospital, Taipei, Taiwan, ROC
8Department of Clinical Laboratory, MacKay Memorial Hospital, Taipei, Taiwan, ROC
9College of Medicine, Chang Gung University, Taoyuan, Taiwan, ROC
10Department of Pediatrics, Chang Gung Memorial Hospital, Taoyuan, Taiwan, ROC

Abstract
Human Leukocyte Antigen (HLA)-DQ2 and HLA-DQ8 are genetic risk factors for Type 1 Diabetes Mellitus (T1DM) and Celiac disease (CD) in Caucasians, but their association with Taiwanese Han population is unknown. We screened 532 Taiwanese T1DM patients for CD biomarkers including anti-tissue transglutaminase (TGM2), anti-gliadin and anti-neoepitope antibodies (Abs), sequencing DQB1 genotypes, and characterized the TGM2 Abs. We report that 3.76% of Taiwanese patients had TGM2-Abs and all had no CD’s symptoms. In contrast to Caucasian’s CD patients, DQ2/DQ8 only constituted ~4/5 of TGM2-Abs positive patients, while the other ~1/5 patients belonged to different HLA genotypes. Either anti-gliadin or anti-neoepitope Abs coexisted with ~3/4 of TGM2-Abs positive patients that were likely due to gluten-ingestion, while the cause of TGM2-Abs production for other ~1/4 of patients was unknown. Purified anti-TGM2 IgA (TGA) and anti-TGM2 IgG (TGG) could bind on endothelial cells surface, recognized native better than denatured forms of TGM2.

Abbreviations: Abs, antibodies; CD, celiac disease; HLA, human leukocyte antigen; IgA, Immunoglobulin A; IgG, Immunoglobulin G; T1DM, type 1 diabetes mellitus; TGA, anti-TGM2 IgA; TGase, transamidation activity; TGG, anti-TGM2 IgG; TGM2, tissue transglutaminase (or transglutaminase 2); TGM2-Abs, TGM2 antibodies.
INTRODUCTION

Celiac disease (CD) is a common autoimmune disease that occurs in genetically predisposed individuals who develop autoantibodies to tissue transglutaminase (TGM2) after ingesting Gln (Q)-rich gliadin, a good TGM2 substrate.\(^1\) In the general population of Caucasians from Europe, North and South America, the Arab region, India, and Pakistan, about 1%-2% of individuals from each region suffer from CD.\(^2\)-\(^4\) About 5% of patients with type I diabetes mellitus (T1DM) also have CD, which is the highest among autoimmune diseases\(^5\); however, 40%-60% of them have no or only mild CD symptoms.\(^6\) If their CD is not treated, they have higher risks of microvascular comorbidities, impaired bone health, and mortality.\(^6\) Thus, screen patients with T1DM for CD at diagnosis of diabetes and during follow-up have been recommended.\(^7\) Currently, there are no reports on screening for CD in T1DM patients in Taiwan.

TGM2 is the predominant autoantigen in CD and serologic screening of anti-TGM2 IgA (TGA) (or anti-TGM2-IgG; TGG) is the standard test for CD as its specificity in predicting CD.\(^8\) TGM2 deamidates Gln (Q) to Glu (E) in the gliadin which increases its affinity to the HLA-DQ2 or HLA-DQ8 molecules on antigen-presenting cells.\(^1\) Then antibodies against TGM2, gliadin, cross-linked TGM2-gliadin (called neoepitope) and actin develop through unclear mechanisms.\(^1,9\) The epitopes of TGA and the biological effects of TGA have been intensively investigated,\(^8\) while the TGG epitopes have never been investigated. Recently, anti-neoepitope Abs are also being used to screen CD demonstrating that TGM2 not only deamidates gliadin but also crosslinks gliadin to TGM2.\(^9\) Although TGM2-Abs are present in most CD patients, they are also found in non-CD individuals with inflammatory bowel disease, viral infections, or end-stage heart failure.\(^8\) Whether all TGM2-Abs positive patients also carry anti-neoepitope and/or gliadin antibodies requires further validation.

Human Leukocyte Antigen (HLA)-DQ2 (DQB*02:01) and DQ8 (DQ8*03:02) confer the greatest risk of T1DM.\(^10\) Remarkably, more than 90% of CD patients carry HLA-DQ2 and most of the remainder have HLA-DQ8.\(^1\) The occurrence of CD in the absence of the aforementioned DQ alleles is rare in Caucasians.\(^1\) However, the presence of DQ2 and DQ8 does not predict the development of CD, since they are present in 25 to 50% of the general population and the majority of these individuals are latent and risk groups for CD.\(^1,10,11\) No CD cases have been reported in Taiwanese T1DM patients and no studies on the association of HLA and CD have been investigated.

TGM2 is a calcium-dependent enzyme with 687 amino acids and catalyzes three types of transamidation reactions (TGase activity): (1) the first type of reaction catalyzes a crosslinking reaction between a specific γ-glutamyl (Q) containing peptide substrate (Q-substrate) and an ε-amino group from a peptide-bound lysine (K) residue (K-substrate)\(^12,13\); (2) when a K-substrate is not available, TGM2 catalyzes the incorporation of the primary amines/polyamines into Q-substrate (aminylation)\(^12,13\); (3) The third type of reaction is when a K-substrate and amines are not available, Q-substrate is deamidated to a glutamate (E) residue under acidic condition (also called deamidation).\(^13\) The deamidation reaction by TGM2 increases the negative charges of gliadin and enhances its affinity to HLA-DQ2/DQ8 and the production of anti-gliadin-, anti-neoepitope-, and TGM2-Abs.\(^8\) In CD, the generation of neoepitope-Abs could be a combination of type I and/or type III reactions,\(^9\) as they have different pH preferences and might be occurring at different parts of intestinal areas. Whether all Taiwanese TGM2-Abs positive T1DM patients also carry anti-neoepitope and/or gliadin antibodies are unknown.

TGM2 is composed of an N-terminal β-sandwich (domain I), a α/β catalytic core (domain II), a β-barrel 1 (domain III), and a β-barrel 2 (domain IV) domains.\(^14\) The TGase active site is composed of a catalytic triad of C\(^{277}\)-H\(^{335}\)-D\(^{358}\).\(^12,14,15\) The Ala mutation at C\(^{277}\) (C\(^{277}A\)) completely disrupts its TGase activity.\(^12\) TGM2 binds GTP with high affinity.\(^14\) Binding of Ca\(^{2+}\) is essential for TGM2 to acquire a catalytically active “open” conformation.\(^12,16\) The importance of amino acids in binding to GTP including R\(^{580}\) has been described.\(^17\) A mutation at R\(^{580}\) to adenine (R\(^{580}A\)) results in almost complete loss of GTP/GDP binding but retained TGase activity.\(^17\) A rare non-proline cis-peptide bond K\(^{387}\)/Y\(^{388}\) of TGM2 that might play a role in modulating and TGA inhibited TGM2’s transamidation activity by up to 80% but TGG had no effects. Epitope mapping of all TGM2-Abs positive sera demonstrated that TGM2-Abs had heterogeneity in specificities. This is the first study on the differences between Taiwanese Han group and Caucasian in HLA genotypes and properties of TGM2-Abs.

KEYWORDS
autoantibodies, celiac disease, Gliadin, Gluten, HLA-DQB1 genotyping, transglutaminase 2
TGase and GTP binding is also described. The binding of TGM2-Abs to these TGM2 mutants with different conformations is not established and is investigated in this investigation.

This study represents the first investigation on the prevalence of TGM2-Abs, anti-gliadin, and neoepitope-Abs, HLA-DQB1 genotypes, and epitopes of TGM2-Abs in Taiwanese T1DM patients. We epitope-mapped the TGA (and TGG) with native and denatured form of TGM2, and several TGM2 mutants. The results revealed that there were distinct specificity between TGA and TGG and heterogeneity among different patients. All TGA (and TGG) positive patients did not show clinical symptoms associated with CD and ~3/4 of our patients had either anti-gliadin and/or anti-neoepitope Abs, demonstrating gluten-containing foods were the triggering factor for these patients. However, ~1/4 of TGM2-Abs positive patients did not have anti-gliadin and/or anti-neoepitope Abs, suggesting that other factors triggered the production of TGM2-Abs in these patients.

2 | RESEARCH DESIGN AND METHODS

2.1 | T1DM patients

A total of 532 Taiwanese Han T1DM patients (239 males/293 females) from the MacKay Memorial Hospital not on gluten-free diets participated in the study. The mean ± SD age at diagnosis was 8.1 ± 4.1 (range, 0.9-18.0) years. The mean T1DM duration was 11.4 ± 7.6 years. The Institutional Review Board approved the study and the patients and/or their guardians gave informed consent.

2.2 | Genotyping of the HLA-DQB1 gene

White blood cells were isolated from TGM2 Ab-positive patients and their DNAs were isolated with Invitrogen's DNA kit. HLA-DQB1 gene was genotyped using SeCore DQB1 Locus Sequencing Kits (Invitrogen/Life Technologies, WI) on an ABI 3730XL DNA Analyzers (Applied Biosystems) with a TYPE6.0 SBT software (Invitrogen).

2.3 | Total IgA ELISAs

Patients’ IgA concentrations were quantified using IgA Human ELISA Kit (Invitrogen, Thermo Fisher, CA) according to human IgA standards provided with the kit. All measurements were performed and presented as the average of two triplicate experiments.

2.4 | Screening of TGA/TGG Antibodies

The 96-well TGA or TGG ELISA plates (ZediXclusive Open ELISA TGA/or TGG kit, Zedira, Germany) were coated with human recombinant TGM2 in the open conformation. The kits came with positive TGA (or TGG) control serum (from Germany) for comparison and normalization. The color was developed using TMB substrate and quantified by VersaMax Microplate reader (Molecular Devices, USA) using OD₄₅₀nm and subtracting OD₆₂₀nm. Based on the manufacturer's instruction, the threshold level ratio of normal (negative) is less than 0.90; equivocal ratio is between 0.90 and 1.12 and positive ratio is more than 1.12. All measurements were performed and presented as the average of two duplicate experiments.

2.5 | Anti-gliadin and anti-neoepitope antibodies ELISAs

Sera (diluted 1:100) was tested for anti-gliadin (IgA and IgG) using purified gliadin (Fitzgerald Industries International, MA, USA) or neoepitope coated 96-well plates or anti-neoepitopes (IgA and IgG) assays (AESKU DIAGNOSTICS, Germany’s CD patients), according to the manufacturer's procedures. All measurements were performed and presented as the average of two duplicate experiments.

2.6 | Affinity purification of TGA and TGG antibodies

As patient # 12 had the highest titer for TGA and IgG, his serum was used for the purification of TGA (or TGG). Total IgAs were first purified using Jacalin conjugated-Sepharose (G-Biosciences, USA,) and the flow through was used for the purification of total IgG using Protein A-Sepharose 4B (Thermo Fisher Scientific, USA). To purify TGM2-specific Abs, total IgA (or IgG) was further purified through the TGM2 conjugated Sepharose column. For the conjugation of TGM2 to Sepharose resins, GST-TGM2 was pre-treated with 1 µM of ZDON, an irreversible inhibitor of TGM2 reported to lock TGM2 in an open conformation, before conjugated to cyanogen Bromide (CNBr)–activated-epharose®4B (Sigma-Aldrich, USA). The open conformation of TGM2 was reported to have stronger binding to TGA. The purified TGA and TGG were verified with SDS-PAGE, Coomassie blue staining, and immunoblotting. In accord with previous reports, the yield of purified TGA was much lower than TGG. ELISAs were performed to ensure the correctness of purified TGA (or TGG).
2.7 | Epitope mapping of TGA (and TGG)

To analyze the specificities of TGA (or TGG) in binding to native or denatured TGM2 (by heating at 95°C for 10 minutes), wells were coated with various amounts of native TGM2 (or denatured TGM2) in 50 mM Tris-Cl pH 7.5, 100 mM NaCl, and 1 mM EDTA (TBS/EDTA) for overnight (O/N). The plates were blocked 1 hour with 3% Bovine Serum Albumin (BSA) in TBS and 0.05% Tween20 (TBST). After blocking, purified patient’s #12 TGA (or TGG) (1:2000) in 1% BSA/TBST or another patient’s serum (1:100) in TBST with 1% BSA was added and incubated for 1 hour. After washing 3X with TBST, bounded TGA (or TGG) was detected using HRP-conjugated anti-human IgA (Zedira, Germany) or HRP conjugated-donkey anti-human IgG 1:20 000 (Jackson ImmunoResearch, USA) in TBST. The color was developed by adding TMB substrate (Sigma-Aldrich, USA) for 20 minutes and the reaction was stopped with 0.1 M H₂SO₄. The absorbance was determined by reading at OD₄₅₀nm minus OD₆₂₀nm as described above. All measurements were performed and presented as the average of two triplicate experiments.

To analyze the specificities of TGA or IgG in binding to TGM2 and different mutants, 8 ng/µL of TGM2 (or R580A, T386A, K387A, D389A, C277A, and triple mutant T386A/K387A/D389A) was coated on a 96 well plate at 4°C O/N. After blocking with BSA, purified TGM2 Abs (1:2000 dilution) in 1% BSA/TBST was added and incubated for 1 hour. After washing, bounded TGA/TGG was detected by incubating 1 hour with HRP conjugated with anti-human IgA (Zedira, Germany,) or HRP conjugated with donkey anti-human IgG (1:20 000) (Jackson ImmunoResearch, USA) in TBST. The color was developed and quantified as described above. All measurements were performed and presented as the average of two triplicate experiments.

2.8 | Small molecules in modulating TGM2 in binding to TGA/IgG

Plate wells were coated with 0, 0.25, 0.5, 1, 2, 4, and 8 ng/µL of TGM2 in TBS containing 2 mM Mg²⁺, 1 mM Ca²⁺, 1 µM Zn⁺², 1 µM ZDON, or 1 µG TGP at 4°C O/N. After blocking with BSA, purified TGM2 Abs (1:2000 dilution) in 1% BSA/TBST was added and incubated for 1 hour. After washing, bounded TGA/TGG was detected by incubating 1 hour with HRP conjugated with anti-human IgA (Zedira, Germany,) or HRP conjugated with donkey anti-human IgG (1:20000) (Jackson ImmunoResearch, USA) in TBST. The color was developed and quantified as described above. All measurements were performed and presented as the average of two triplicate experiments.

2.9 | Construction of recombinant TGM2 and mutants

All mutants were constructed using the quick-change site-directed mutagenesis kit (Agilent technologies, CA) and verified by DNA sequencing. TGM2 active site mutant C277A was constructed previously. In addition, non-proline cis-peptide mutants including T386A, K387A, and D389A and 386AAXA389 triple mutants (designated as GTP4) were also constructed. The purification recombinant GST-TGM2 and mutants in *Escherichia coli* are as described.

2.10 | Purification of recombinant TGM2 and mutants in *E coli*

Purification of wild-type TGM2 and mutant proteins, the conditions for growing *E coli* harboring either the TGM2 or TGM2/C277A vectors, and the purification of glutathione-S-transferase (GST) fusion proteins were as previously described. Final purified recombinant proteins were dialyzed against 50 mM Tris-acetate, pH 8, 1 mM DTT/EDTA, 20% glycerol, and stored in aliquots at −20°C. All affinity purified proteins migrated in the SDS-polyacrylamide gels in accordance with the expected protein size of the construct. In most preparations, the protein was >95% pure based on Coomassie Blue staining.

2.11 | Non-permeabilized immunofluorescence staining

Human umbilical cords endothelial cells (HUVEC) cells were grown on a 0.2% gelatin-coated cover glass in EGM-2 media (Lonza). After washed with warm Hanks Balanced Salt solution, cells were fixed with 4% paraformaldehyde in PBS for 30 minutes at room temperature. Cells were blocked with 1% BSA in PBS for 30 minutes at room temperature, the purified TGA or TGG was used as a primary antibody (1:100 in 1% BSA in PBS). Bound TGA or TGG was detected using goat anti-human IgA conjugated with FITC (1:50) (Jackson ImmunoResearch, USA), or donkey anti-human IgG conjugated Alexa594 (1:100) (Jackson ImmunoResearch, USA), respectively. Nucleus was counter-stained with Hoesche33342 (Sigma-Aldrich, USA). The cover glasses were sealed on the slide with mounting media (Antifade Mount, Thermo Fisher Scientific, USA). All cell stainings were performed for at least two repeated experiments.

2.12 | Transamidation reaction, 5-(biotinamido)pentylamine-incorporation assay

The assay was performed using N, N′-dimethylcasein coated on a 96-well plate for O/N as described. All measurements were performed and presented as the average of two triplicate experiments.
### 2.13 Data and resource availability

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

### 3 RESULTS

#### 3.1 Screening of TGM2-Abs in Taiwanese T1DM patients

Among 532 T1DM patients, there were 11 with TGA (2.06%), 11 with TGG (2.06%), and 2 with both TGA and TGG (0.38%), and 20 with either TGA or TGG (3.76%) (Tables 1 and 2). All TGM2-Abs positive patients can be grouped into 77.8% (~4/5) with either DQ2 or DQ8, and can be sub-grouped into 11.1% of DQ2/DQ2 (2/18); 5.6% of DQ8/DQ8 (1/18); and 22.2% of DQ2/DQ8 (4/18). Total human IgA concentrations were quantified in patients with TGG, and their IgA were found to range from 0.8 to 3.8 mg/mL.

TGM2-Abs positive patients were given questionnaires (based on the Celiac disease foundation’s criteria; Celiac. Org) for any clinical symptoms associated with CD and all reported with no persisting symptoms including abdominal pain, acid reflux (heartburn), bloating, constipation, diarrhea, gas, lactose intolerance, pale foul-smelling stool, or unexplained liver problem. They had never been diagnosed with lymphoma or intestinal cancer. Therefore, intestinal endoscopic examinations were not performed. Their mean age at this study was 15.7 ± 7.7 (range, 5.2-29.9) years.

#### 3.2 Screening for anti-gliadin and anti-neoepitope Abs

To investigate the triggering factors for TGM2-Abs production, commercial anti-gliadin and anti-neoepitope Abs kits were used (Table 3). All samples and positive controls provided by the kits were performed in duplicate. Similar to the TGA/TGG result, patient 12’s serum was the only patient to have both anti-gliadin and anti-neoepitope IgA/IgG and were the highest titers (Table 3). Around ~3/4 (74%) of TGM2-Abs positive patients were found to associate with either anti-gliadin or anti-neoepitope Abs (Table 3). Both anti-gliadin and anti-neoepitope Abs were not detected in ~1/4 (26%) of TGM2-Abs positive patients (Table 3).

#### 3.3 Purified TGM2-Abs recognized native better than denatured TGM2

As patient #12 had the highest TGM2-Abs titers, his serum was used for the purification of TGA/TGG. We first

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**TABLE 1** T1DM patients with TGA (or TGG) and their respective HLA genotypes

| Patient ID | 12  | 5982 | 357 | 5901 | 6022 | 6028 | 6119 | 6214 | 6340 | 6569 | 6636 |
|------------|-----|------|-----|------|------|------|------|------|------|------|------|
| TGA (Fold*) | 4.5 | 3.6  | 2.8 | 1.0  | 1.4  | 1.5  | 1.5  | 1.4  | 1.7  | 1.1  | 1.2  |
| TGG (Fold*) | 1.7 | 1.4  | –   | –    | –    | –    | –    | –    | –    | –    | –    |
| DQB1 allele 1 | 02:01 | 03:02 | 02:01 | 02:01 | 02:01 | 02:01 | 02:01 | 02:01 | 02:01 | 03:01 | NA  | NA  |
| DQ Type | DQ2 | DQ8 | DQ2 | DQ2 | DQ2 | DQ2 | DQ2 | DQ2 | DQ7 | NA | NA |
| DQB1 allele 2 | 03:02 | 03:02 | 03:02 | 04:01 | 03:03 | 06:01 | 03:02 | 03:03 | 03:03 | NA | NA |
| DQ Type | DQ8 | DQ8 | DQ8 | DQ9 | DQ9 | DQ9 | DQ9 | DQ9 | NA | NA |

**Note:** NA, not available. All measurements were presented as the average of two duplicate experiments.

*Based on the company’s instruction manual, patients with the Ab levels above the threshold ratio of 1.12 is considered as TGA (or TGG) positive. Therefore, the “fold” is calculated based on (patient’s Ab response)/(1.12). If they are below 1, the fold is labeled as “–”.

**TABLE 2** Patients with TGG and their respective HLA types

| Patient ID | 190 | 423 | 5793 | 5828 | 5898 | 6026 | 6054 | 6101 | 6357 |
|------------|-----|-----|------|------|------|------|------|------|------|
| TGM2-IgA (Fold*) | – | – | – | – | – | – | – | – | – |
| TGM2-IgG (Fold*) | 1.0 | 1.3 | 1.9 | 1.6 | 1.1 | 1.7 | 1.2 | 1.6 | 1.3 |
| DQB1 allele 1 | 02:01 | 03:03 | 02:01 | 03:02 | 03:01 | 03:03 | 03:02 | 02:01 | 02:01 |
| DQ Type | DQ2 | DQ9 | DQ2 | DQ8 | DQ7 | DQ9 | DQ8 | DQ2 | DQ2 |
| DQB1 allele 2 | 03:03 | 04:01 | 02:01 | 03:03 | 06:02 | 04:01 | 04:01 | 03:03 | 02:01 |
| DQ Type | DQ9 | DQ4 | DQ2 | DQ9 | DQ6 | DQ4 | DQ4 | DQ8 | DQ2 |

*Based on the company’s instruction manual, patients with the Ab levels above the threshold ratio of 1.12 is considered as TGM2 Abs positive. Therefore, the “fold” is calculated based on (patient’s Ab response ratio)/(1.12). If the fold is below 1, it is labeled as “–”. All measurements were presented as the average of two duplicate experiments.
investigated whether purified TGM2-Abs can bind to native or denatured TGM2. As shown in Figure 1, purified TGA was found to bind <5% of denatured TGM2 (Figure 1A), while purified TGGs were shown to bind up to 20% of denatured TGM2 (Figure 1B). To demonstrate that denatured TGM2 was not precipitated out of the solution during the denaturing process, mouse monoclonal TGG (CUB7402, Thermo Fisher Scientific) was found to bind denatured TGM2 almost to the same extent as native TGM2 (Figure 1C), suggesting the denatured TGM2 was not precipitated during denaturing process.

3.4 | Sera from other positive TGA (and TGG) patients in recognizing native and denatured TGM2

Sera from other TGA positive patients and positive control patients from the kit was also investigated. Similar to purified #12’s TGA, kit’s positive control serum bound native TGM2 much better than denatured TGM2 (Figure 2A). However, sera obtained from five other patients (6119, 6028, 6214, 6340, and 6569) recognize 20% - 26% of denatured TGM2 compared to native TGM2 (Figure 2A). Similarly, sera obtained from patients 6636 and 6022 were found to bind ~ 50% of denatured TGM2 (Figure 2B), while sera obtained from patient 5901 and 357 were found to bind denatured TGM2 much better than native TGM2 (Figure 2B). These data demonstrated that there were heterogeneities of TGA’s epitopes in Taiwanese T1DM patients. Other TGG positive patients (423, 6016, and 5828) bound equally well to native and denatured TGM2, while Kit’s positive control bound native better than denatured TGM2 (Figure 2C).

### 3.4.1 Epitope mapping of TGA/TGG

Non-proline cis-peptide bonds are rare and normally play an important role in protein’s structure and function. To investigate whether point mutation on a unique non-proline cis-peptide bond K387/Y388 of TGM2 affects the antibody binding (Figure 3A), we mutated selected key residues in 386TKXD389 to T386A, K387A, D389A, and GTP4, a triple mutant. In addition, active site mutant, C277A, and the GTP binding site mutant R580A were also investigated (Figure 3A) These point-mutation mutants were coated on a 96-wells plate and the binding ability of TGA (or TGG) was determined.

For convenience, we grouped different TGA positive patients into Figure 3B to Figure 3D based on their similarity in binding to different mutants. When comparing the binding to TGM2 and mutants, purified patients 12’s TGA, and sera from 6340’s and 6569’s were found to bind only <50% of T386A. However, purified patient 12’s TGA was ~twofold better than TGM2 in binding to R580A, K387A, D389A, and GTP4 mutants, suggesting that these four mutants may have exposed different conformations. These data suggested that there were unique TGA epitopes among patients 12, 6340, and 6569 (Figure 3B).

### Table 3

(A) The antibody (Ab) response to gliadin and neoepitope from serum with TGA positive patients. (B) The antibody response to gliadin and neoepitope from serum with TGG positive patients

| ID # | 12 | 357 | 5901 | 5982 | 6022 | 6028 | 6119 | 6214 | 6340 | 6569 | 6636 |
|------|----|-----|------|------|------|------|------|------|------|------|------|
| Anti-Gliadin Ab | IgA (fold*) | 6.7 | – | – | – | – | – | – | – | – | 1.0 |
| | IgG (fold*) | 3.5 | – | – | 3.4 | 5.1 | 1.5 | 1.8 | 3.0 | 3.9 | 1.7 | – |
| Anti-Neo-epitope Ab | IgA (fold*) | 170.1 | 3.6 | – | 3.4 | 2.2 | 1.3 | 2.0 | – | 4.2 | 1.7 |
| | IgG (fold*) | 5.5 | – | – | 2.3 | 1.2 | – | – | – | – | – |

| ID # | 190 | 423 | 5793 | 5828 | 5898 | 6026 | 6101 | 6357 | 383a |
|------|-----|-----|------|------|------|------|------|------|------|
| Anti-Gliadin Ab | IgA (fold*) | – | – | – | – | 1.1 | – | – | – |
| | IgG (fold*) | 3.3 | 1.5 | – | – | 1.1 | 3.2 | – | – |
| Anti-Neo-epitope Ab | IgA (fold*) | – | – | – | – | – | – | – | – |
| | IgG (fold*) | 1.3 | – | – | – | – | – | – | – |

a*Based on the company’s instruction manual, the threshold levels of anti-gliadin Ab and anti-neoepitope Ab is ≥12, or 18 U/mL, respectively. Therefore, the “fold” is calculated based on (patient’s Ab concentration)/(12 or 18, respectively). If the fold is below 1, it is labeled as “–”. All measurements were presented as the average of two duplicate experiments.

b#The T1DM patient # 383 was TGA/TGG negative and was used as a negative control.
In Figure 3C, TGA positive patients 6119, 6028, 6214, and commercial kit’s positive control were analyzed. kit’s TGA positive control represents serum derived from Caucasian CD patient. These patients bound similarly to all mutants with <50% to T386A and 100%-140% to R580A, K387A and D389A mutants.

In Figure 3D, four TGA positive patients’ sera (6636, 6022, 357, and 5901) were compared based on their relatively high binding (50%-140%) to denatured TGM2. Similar to purified 12’s TGA in Figure 3B, patient 357’s serum had ~twofold higher in binding to R580A, K387A, and D389A mutants.

In summary, TGA positive patients had increased binding to K387A, D389A, and R580A (9 out of 11 patients or 9/11) and reduced binding to C277A (7/11) and T386A (9/11) (Figure 3A), while positive TGG patient’s sera had reduced binding to C277A (4/4) and D389A (4/4) (Figure 3A). The variations of sera’s TGA and TGG in binding to different TGM2 or mutants represent there were heterogeneity of the polyclonal epitopes for the TGA and TGG.

3.5 | Small molecules in modulating the binding of purified TGA or TGG to TGM2

In Figure 4A, the binding of purified TGA to TGM2 pretreated with various small molecules was analyzed and all was shown to have increased in binding in the following orders: Mg$^{2+}$ (171.7%) > GTP (153.5%) > ZDON (147.6%) > Zn$^{2+}$ (147.1%) > Ca$^{2+}$ (126.5%) (Figure 4A). In contrast, TGM2 pretreated with these small molecules did not change their binding to the purified TGG (Figure 4B). These data demonstrated that certain small molecules could increase the binding of TGA but not TGG.

FIGURE 1 Purified TGM2-Abs in recognizing the native and denatured form of TGM2. The ELISA was performed as described under Materials and Methods. Wells of a 96-well plate were coated O/N with 0.25, 0.5, 1, 2, 4, or 8 ng/µL in TBS/EDTA of native and denatured TGM2. After blocking, purified TGA (1:2000) (A) or TGG (1:2000) (B) in 1% BSA/TBST were added and incubated for 1 hour. Bound TGA (A) or TGG (B) was detected with anti-human IgA conjugated with HRP or donkey anti-human IgG conjugated with HRP (1:20 000), respectively. (C), Mouse anti-TGM2 monoclonal antibody (CUB7402) was used as 1st antibody and goat anti-mouse IgG conjugated HRP was used as 2nd antibody. Data were performed in two triplicates and are presented as means ± standard deviations.
3.6 | Small molecules in modulating the binding of purified TGA or TGG to K387ATGM2

As shown in Figure 4C, K387A mutant was shown to have the highest ability in binding to TGA and we were interested in knowing whether the binding of TGA could be further enhanced to K387A pretreated with these small molecules. K387 mutant pretreated with these small molecules did not change the binding of purified TGA to K387A (Figure 4C). These data demonstrated that the K387A was in a conformation that had the highest in binding to TGA.

3.7 | Effects of purified TGA (and TGG) on the transamidation (TGase) activity of TGM2

Due to there was difficulty in purifying large amount of TGA, the concentration of purified TGA used in the assay was smaller than when compared to purified TGG. As the transamidation activity assay requires the presence of DTT to display optimal activity, we used a minimal DTT concentration (0.5 mM) in the reaction to minimize its potential effects on IgA (or TGG)'s structure (Figure 5).

There were dosage-dependent effects of purified TGA in inhibiting TGase activity, but the inhibition was not complete as there was 20%-30% residual TGase activity. The IC50 of the TGA/TGM2 ratio in inhibiting TGase activity was ~2, (Figure 5A). In contrast, purified TGG had no effects on TGM2’s activity (Figure 5B).

3.8 | Effects of fibronectin in modulating the binding of purified antibodies in binding to TGM2

Previous studies demonstrated that there are four monoclonal TGA epitopes on TGM2 and one of these epitopes is located at
FIGURE 3  Sera obtained from TGA (or TGG) positive T1DM patients in binding to TGM2 and its mutants. A, The structural and functional domains of TGM2 together with the locations of the mutations are illustrated. The results obtained from B-E are summarized with the number of patients with increased or decreased binding to TGA (or TGG) are also indicated. B-E, Recombinant TGM2 (8 ng/µL in TBS/EDTA) was coated on a 96-well plate for O/N. After blocking and incubating with sera from nine different T1DM patients with TGA (B-D) or three patients with TGG (E) (1:100 dilutions), bound TGA (or TGG) were detected with either anti-human IgA conjugated with HRP (B-D) or donkey anti-human IgG conjugated with HRP (E). Purified TGA/TGG (1:2000) and commercial kit’s positive control were used for comparison. The bound TGA/TGG was quantified as described under Materials and Methods. The binding to TGM2 was used as 100% and normalized to other groups. Data were performed in two triplicates and are presented as means ± standard deviations.
To investigate whether purified TGA (or TGG) can compete with FN on binding to TGM2, we pre-incubated increased concentrations of FN and purified TGA (or TGG) in solution before applying to the TGM2 coated plate (Figure 6). Our results also showed FN had no significant effect in competing with purified TGA (or TGG) in binding to TGM2 (Figure 6).

3.9 | Purified TGA/TGG bind on the surface of HUVEC cells

HUVEC is commonly used vascular endothelial cells and TGM2 was reported to express on the surface of HUVEC. Immunostaining demonstrated that purified TGA/TGG could bind to the surface of the HUVEC (Figure 7).

4 | DISCUSSIONS

As TGM2 is an autoantigen for CD and the TGA and TGG are widely used as the standard screening tests for CD due to its specificity and inexpensiveness, we screened TGA/TGG for potential CD in our T1DM patients. The ingestion of gluten also generates anti-gliadin and anti-neoepitope Abs in CD patients. Therefore, the positive anti-gliadin and/or anti-neoepitope Abs in addition to TGA (or TGG) indicate that gluten-ingestion is the triggering factor in our patients.
However, since TGA (or TGG) is also detectable in some patients with other chronic inflammatory conditions including viral infections, inflammatory bowel disease, and end-stage heart failure, the absence of anti-gliadin and neo-epitope Abs differentiates them from gluten-ingestion. Additionally, the epitopes of TGM2-Abs derived from non-CD patients are distinct from those from CD patients, suggesting that the production of TGM2-Abs might be triggered by different mechanisms. In this study, we also found the differences in TGA (or TGG) epitopes between our patients and Caucasian CD patients.

T1DM ranks the highest among autoimmune diseases in risk to be associated with CD. In Caucasians, the prevalence of CD is ~5% in T1DM patients and 3-4 times higher than in the general population. The prevalence of TGM2-Abs was 3.76% (20/532) in our patients but they had no clinical symptoms of CD. Therefore, our TGM2-Abs positive patients should be classified as the latent form of CD. The majority (~3/4) of our TGM2-Abs positive patients were also associated with either anti-gliadin and/or anti-neoepitope antibodies (Table 3), indicating that TGM2 Abs production was caused by gluten ingestion. However, ~1/4 of TGM2-Abs
positive patients did not have anti-gliadin and/or anti-neoeptope Abs, suggesting that other uncharacterized chronic conditions were triggering the production of the Abs (Table 3). Our results also indicated that the presence of TGM2-Abs had better predictive value for potential CD as 26.3% of TGM2-Abs positive patients did not have either anti-gliadin Abs and/or anti-neoepitope Abs (Table 3). For patients with three different Abs, patient #12 is unique in that his anti-neoepitope Abs titers were much high than the combined titers of anti-gliadin and TGM2-Abs (Tables 1 and 3). The results are consistent with previously described.26 Competition assay by preincubating serum with recombinant TGM2 was still found to bind neo-epitope plate, indicating the epitope for TGM2-Abs was distinct from anti-neoepitope Abs (data not shown) and is consistent with Lerner et al reported.26 This raises an important question of the biological effects of anti-neoepitope Abs. As the generation of neo-epitope is the combination of gliadin peptide-TGM2,9,26 we would expect to find the co-appearance of anti-gliadin and anti-neoepitope Abs in patients. However, there were four patients (6214, 6569, 5793, and 6101) with anti-gliadin IgG but did not have anti-neoepitope Abs (Table 3). The data suggest that there were distinct epitopes for anti-gliadin and anti-neo-epitope. Conversely, one patient (357) had anti-neoepitope IgA but did not have anti-gliadin Abs (Table 3). As TGase-catalyzed reactions have different pH preferences and occurring at different intestinal areas and may contribute to the different Abs responses in these patients.

For those with TGG (Tables 1 and 2), their total serum IgA levels were at the lower range of normal IgA concentrations, suggesting IgA deficiency in these patients. As selective IgA deficiency in T1DMs is relatively common and might be explaining higher percentage of TGG in our patients.32 In Caucasians, almost all CD patients carry homozygous DQ2 or DQ8 or heterogeneous DQ2. Overall, 77.8% (~4/5) of Taiwanese T1DM patients had at least one DQ2 or DQ8 genotype, which is lower than Caucasians but DQ2/ DQ8 still was the most susceptible genotypes in our patients. Our results showed that DQ9 as the other susceptible genotype, which has not been reported in Caucasians with CD. In screening adult Chinese patients with diarrhea irritable bowel syndrome, Wang H et al described DQ 9.3 as a susceptible genotype for CD in the Chinese population.29 In addition, we also found that four TGM2-Abs positive patients had unique genotypes including DQ7/DQ6 (TGG), DQ7/DQ9 (TGA), and DQ9/DQ4 (TGG) which have not been reported in Caucasians with CD.33-39

The purified TGM2-Abs were characterized based on several criteria including: (1) the ability to bind native and denatured TGM2; (2) the ability to bind to different TGM2 mutants; (3) the ability to inhibit transamidation activity; (4) the ability to compete with FN in binding to TGM2; and (5) the ability to bind on the endothelial cell surface. Both purified TGM2-Abs bind to native better than the denatured form of TGM2 (Figure 1), suggesting that these antibodies recognize a native conformation. Similar results were also found in IgA from the other six Taiwanese patients (Figure 2A) and positive control's IgA provided by the kit which represent serum from Caucasians with CD (Figure 2A). In consistent with this finding, purified

**FIGURE 7** Immunofluorescence staining of purified TGA (or TGG) in binding to endothelial cell’s surface. Human umbilical cord endothelial cells (HUVEC) were grown on 0.2% gelatin-coated cover glasses. Cells were fixed with 4% paraformaldehyde in PBS for 30 minutes at room temperature. After blocking, the purified TGA (or TGG) (1:100 dilution) was used as the 1st antibody. A, Bound TGA was detected with a goat anti-human IgA conjugated with FITC (1:50 dilution). D, Bound TGG was detected with a donkey anti-human IgG antibody conjugated with Alexa594 (1:100 dilution). B and E, Nuclei were counter-stained with Hoesche33342. The images were photographed with 400 X fluorescence microscope. Data were performed in two separate staining experiments.
antibodies also could not detect SDS denatured-TGM2 on a western blot (data not shown). Our data are consistent with previous results using CD patient-derived TGA cannot bind to urea denatured-TGM2. However, two of our patient's TGA bound denatured better than native TGM2, and two other ones bound only half of denatured TGM2, suggesting heterogeneity in recognizing the native or denatured form of TGM2 (Figure 2B).

For TGG patients, the difference in recognizing native or denatured TGM2 was not that great (Figure 2C). Purified TGG and kit's positive control serum recognized native TGM2 with twofold better than denatured TGM2. However, the other three patients recognized almost equally to native and denatured TGM2 (Figure 2C). The data indicate that there are different epitopes of TGA and TGG in recognizing native and denatured TGM2.

Point-mutation mutants were used to investigate the variations of either purified TGA (or TGG), positive control from the kits, or sera from other patients in binding to these mutants. Point-mutation mutants located in a rare non-proline cis-peptide fragment inside the 386TKYD389 of TGM2 were used (Figure 3A). Point mutation to alanine could change the structure and conformation, enzyme activity, and GTP binding ability of TGM2 and will be investigated elsewhere. However, these mutants were useful in locating the precise location of the epitopes of the TGA (and TGG). Our data indicated that TGA and TGG had different specificities toward TGM2 mutants (Figure 3A). In TGM2-Abs derived from Caucasian's CD patients, contradictory results exist on the epitope. Studies from Ireland found most CD and Dermatitis herpetiformis patient's IgA did not bind to catalytic triad triple mutants (C277A/H335A/D358A), while serum's IgG had similar binding to both wild type and catalytic triad mutants of TGM2, suggesting different specificities against TGM2 (or mutants). Studies from Hungarian's CD patients found E153/E154 and R19 are important for the epitopes and demonstrates that the epitopes of TGA derived from CD patients are different from other autoimmune diseases. Here, we found the epitopes of the positive control patient's TGA from the kit were similar to three but different from six of our patients (Figure 3C). The data indicate that there were heterogeneities of TGM2-Abs epitopes in Taiwanese patients.

Various TGM2's activities including TGase, GTP binding, and hydrolysis are regulated by Ca\(^{2+}\), Mg\(^{2+}\), Zn\(^{2+}\), GTP, and ZDON. The binding of small molecules to TGM2 can induce a different conformation and display different TGM2 activities. Calcium (Ca\(^{2+}\)) induces TGM2 to an active conformation for TGase activity. ZDON binds and locks TGM2 to an open active TGase conformation. GTP induces TGM2 to an inactive compact conformation. Mg\(^{2+}\) is required to display TGM2's GTP hydrolysis activity. Zn\(^{2+}\) can inactivate TGM2's TGase activity. These small molecules all can induce TGM2 to a conformation resulted in an increasing binding to TGA but had no effects on TGG, indicating different specificities between purified TGA and TGG (Figure 4A,B). In consistent, use serum derived from CD patients, Ca\(^{2+}\) was found to increase TGM2 in binding to TGA. Other small molecules especially Mg\(^{2+}\) can increase binding of TGA to TGM2 by up to 180% has not been reported (Figure 4A). Both ZDON and GTP induced-TGM2 resulted in an increasing binding to TGA, but had no effects on TGG (Figure 4A,B). As the K387A mutant already had a two-fold increase in binding to purified TGA, pretreated with these small molecules had no further effects on the binding of TGA (Figure 4C).

Purified TGA, but not TGG, inhibited > 80% of transamidation activity (Figure 5). The remaining 20% activity could still have an impact on in vivo biology and warrant further investigations. Although the effects of purified TGA (or TGG) on transamidation activity have been reported, the results are not consistent due to the purity of the Abs used in their assays. Using purified total IgA (or IgG) from six CD patients, either total IgA or IgG from each patient can inhibit 11%-39% of TGM2's transamidation activity in vitro. Using the TGM2-affinity column purified TGA from a group of CD patients, Dieterich et al observed a 40%-50% inhibitory effect on TGase activity. Our data are consistent with the literature that TGA can inhibit the transamidation activity of TGM2, despite our purified TGA and TGG were different in epitopes from Caucasian's CD patients and might explain their differential ability in inhibiting transamidation activity.

Fibronectin (FN) was shown previously to compete with one of the monoclonal TGA derived from CD patient in binding to TGM2 but not in our study (Figure 6). In Caucasian, the epitope 1 monoclonal TGA accounts for about 20% of their total monoclonal Abs. Using total IgA or IgG from CD patients, Király et al conducted similar experiments with FN competing with TGA in binding to TGM2. However, we used TGM2-affinity purified Abs had less interference and our TGA/TGG also had different epitopes. As we only used purified Abs from Patient #12, it remains to be determined whether other patient's TGM2-Abs have similar characteristics.

Both purified TGM2-Abs bound on the endothelial cell surface (Figure 7). The biological effects of TGA derived from CD patients have been reported as described below. TGM2-Abs from CD patients can inhibit epithelial cell differentiation and increase its proliferation. CD patients derived antibodies could recognize TGM2 and increase epithelial permeability and activate monocytes and disturb angiogenesis in vitro. The destruction of the angiogenesis may lead to the damage of the mucosal vasculature in CD in vivo leading to mucosal flattening. Given the fact that the epitopes are different from those derived from Caucasian's CD patients, we would expect they would have different biological effects and warrant further investigation.

In this study, we studied 552 of T1DM patients from a major medical center in northern Taiwan. Standard clinical
practice to confirm the diagnosis of CD requires serological testing, CD symptoms, and endoscopic biopsy. Since none of our TGM2-Abs positive patients had clinical symptoms of CD., there was no justification for endoscopic biopsy. One potential factor for lack of symptoms is that most of these patients were young because aging could be an important factor in the development of clinical symptoms of CD. In Caucasians, 40%-60% of T1DM patients with CD are asymptomatic.6 These could explain why our patients had no clinical CD symptoms. The prevalence of CD in T1DM patients has been reported in 12 studies from Europe, North America, and Australia with sample sizes ranging from 47 to 848.6 Our sample size is more than those in 10 studies.6 Therefore, our results should be significantly representative. The presence of protecting factors against CD in Taiwanese T1DM patients could not be excluded and warrants further investigation.

In conclusion, this is the first comprehensive study on the characterization of the DQB1 genotypes, epitopes, biochemical and in vitro cell binding properties of TGA and TGG derived from Taiwanese T1DM patients. Different from previous studies focusing on TGA, we characterized TGA as well as TGG from our T1DM patients. Our studies revealed several major racial differences as compared to Caucasian's CD patients. In particular, around 1/4 (23.6%) of our TGM2-Abs positive patients could be due to other inflammatory conditions as they do not have anti-gliadin or anti-nectepitope Abs (Table 3). HLA-DQ2 and HLA-DQ8 were not the exclusive genotypes associated with TGM2-Abs production as reported in Caucasian's CD patients. We found DQ9 was the other susceptible genotype for Taiwanese T1DM patients to have TGM2 Abs. Although there were heterogeneities in the epitopes of TGM2-Abs, C277 and T386 appeared critical for the recognition by TGA, while C277 and D389 were important for TGG recognition since point mutation at these sites resulted in the reduced binding of TGA (or TGG) (Figure 3A). We purified TGM2-Abs from one patient for additional characterization. Several small molecules could enhance the TGA in binding to TGM2 but not TGG. Some of these small molecules are abundant in vivo and could play a role in the biology of TGM2-Abs. Purified TGA/TGG also did not compete with FN in binding to TGM2. Around 3/4 of our TGM2-Abs positive patients were related to gluten-ingestion (Table 3), while the other 1/4 was due to unknown inflammatory conditions. The implications of current studies will certainly bring attention to TGM2 Abs screening to the general Taiwanese population to prevent co-occurrence for additional complications. Although our patients had no clinical symptoms, studies have shown asymptomatic CD patients still will be benefited from a gluten-free diet.52

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CONFLICT OF INTEREST
The authors declare no conflict of interest.

AUTHORS CONTRIBUTIONS
T.-S. Lai, C.-J. Lin, and Y.-J. Lee designed the study; W.-H. Ting, Y.-W. Yang, C.-Y. Huang, F.-S. Lo, C.-C. Chu, C.-L. Lin, W.-S. Lin, and Y.-T. Hsieh carried out the experiments and collected the data; T.-S. Lai and Y.-J. Lee wrote the manuscript; Drs. T.-S. Lai and Y.-J. Lee are the guarantors of this work and, as such, had full access to all the data in the study and take the responsibility for the integrity of the data and the accuracy of the data analysis. All authors read and approved the final manuscript.

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