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ORIGINAL PAPER

Genetic variations in the CεmX domain of human membrane-bound IgE

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Abstract The ε chain of membrane-bound IgE (mIgE) is expressed predominantly as a "long" isoform, containing an extra segment of 52 amino acid (a.a.) residues, referred to as CεmX, between the CH4 domain and the C-terminal membrane-anchoring transmembrane peptide. CεmX results from an alternative splicing of the ε RNA transcript at 156-bp upstream of the splicing acceptor site used by the "short" isoform. Here, based on an analysis of the CεmX genomic DNA sequences of 320 subjects residing in Taiwan, we report that single-nucleotide polymorphisms have been found at two positions, namely, G/T at #46 and A/G at #93 (along the 156 bp of CεmX), with the former creating an amino acid change from Val to Leu at #16 (along the 52 a.a. of CεmX) and the latter resulting in no change (Gly). Among the 640 CεmX sequences identified, the previously known 46G93A allelic form appeared 293 times, the newly discovered 46T93A allelic form (GeneBank accession no. GU208817) 26 times, and the 46G93G allelic form (GU208818) 321 times. No 46T93G allelic form was found. Serum IgE measurements showed that the polymorphisms did not correlate with the levels of serum IgE. The anti-CεmX monoclonal antibody, 4B12, could bind equally well to mIgE.Fc_L(16V) and mIgE.Fc_L(16L). While genetic variation of CεmX of broader populations should also be investigated, these newly discovered genetic variants of CεmX in the Taiwanese population do not seem to affect the feasibility of using an anti-CεmX mAb, such as 4B12, to target mIgE-expressing B cells.

Keywords IgE · CεmX · Single-nucleotide polymorphisms · Alleles · Allergy

Abbreviations

BCR	B cell receptor
mAb	Monoclonal antibody
mIgE	Membrane-bound IgE
migis	mIg isotype-specific peptide
migis-ε	Migis peptide of mε chain
SNP	Single-nucleotide polymorphism

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Introduction

The genes of the heavy chain constant (CH) regions of the five classes of human immunoglobulins appear as a cluster in chromosome 14. Apart from an active IGHE gene encoding an ε chain, two ε pseudogenes exist (Max et al. 1982; Nishida et al. 1982). IGHEP1, which is also located in the Ig cluster on chromosome 14, is a truncated IGHE gene (Hisajima et al. 1983; Max et al. 1982); IGHEP2, located inside JAK2 gene on chromosome 9, is a processed ε gene that is flanked by LTR-like elements (Battey et al. 1982; Ueda et al. 1982). Membrane exons are present only in IGHE and IGHEP1 genes (Fig. 1a).

IgE plays a central role in the pathogenesis of allergic asthma and contributes to both the early and late-phase inflammatory cascades of the airway (Gould and Sutton 2008; Oettgen and Geha 2001). In addition to mediating these allergic cascades, IgE increases the number of high-affinity IgE Fc receptors on mast cells and promotes mast cell survival (Kawakami and Galli 2002; Kraft and Kinet 2007). IgE has proven to be a suitable therapeutic target to treat allergic asthma: omalizumab, a humanized anti-IgE antibody, has been approved for the treatment of severe or severe-to-moderate allergic asthma in many countries (Chang et al. 2007; Holgate et al. 2005).

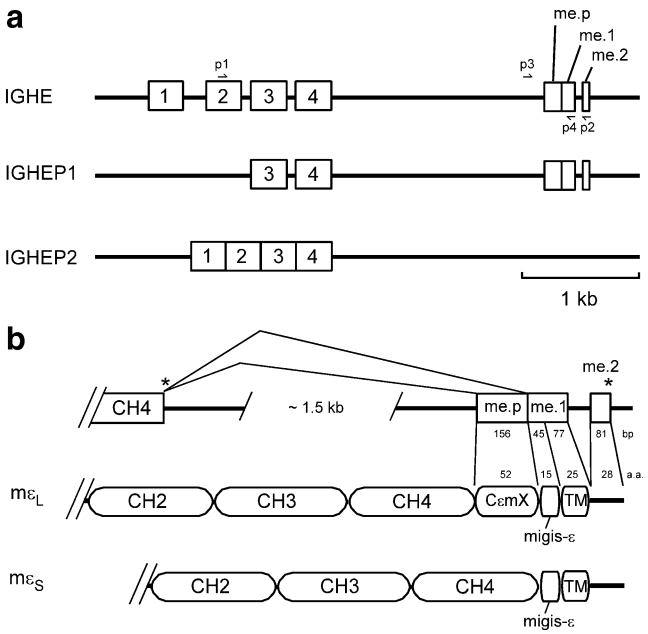

Fig. 1 **a** Schematic representations of the gene structures of IGHE, IGHEP1, and IGHEP2 loci. IGHE contains all of the ε genomic components, and other two are nonfunctional ε pseudogenes. The *boxed* segments represent exons. The *arrows* indicate the segments that the primers used in this study were designed to bind to. **b** Schematic illustration of the alternative splicing of CH4 exon to me.p or me.1 exon in the mε chain RNA transcript, resulting in two mRNA isoforms encoding, respectively, the long and short forms of mε chains. *TM* transmembrane, *asterisk* stop codon

Membrane-bound IgE, which forms B cell receptors (BCRs) with Igα and Igβ, and probably other proteins on mIgE-expressing B cells, is essential for generating isotype-specific IgE responses (Achatz et al. 1997, 2001; Batista et al. 1996). The membrane-anchoring peptide of membrane-bound ε chain (mε), which is not present in the ε chain of secretory IgE, contains a cytoplasmic tail, a transmembrane peptide, and an extracellular "spacer" peptide (referred to as "mIgE isotype-specific" or "migis-ε" peptide (Davis et al. 1991) or "extracellular membrane-proximal domain" or "EMPD" (Batista et al. 1996)). Our group and others have previously found that human mε is present as both long and short isoforms, with the former containing an extra segment of 52 amino acid residues between CH4 and migis-ε peptide (Batista et al. 1995; Peng et al. 1992; Zhang et al. 1992; Fig. 1b). The long isoform is the predominant one produced by IgE-expressing B cells and activated primary B cells (Peng et al. 1992; Zhang et al. 1992). The sequence of CεmX is unique among all known protein sequences; hence, CεmX provides an attractive antigenic site for immunological targeting of IgE-committed B lymphoblasts and memory B cells. Several anti-CεmX mAbs, including a20 (Chen et al. 2002) and others have been generated in our group. The mAb 4B12 binds most strongly to $mIgE^+$ B cells and has been shown to inhibit IgE synthesis in cultures of peripheral blood mononuclear cells (PBMCs) from patients with atopic dermatitis (Chen et al. 2010), suggesting that 4B12 can potentially be used for targeting mIgE-expressing B cells to down-regulate IgE production. To assess the applicability of anti-CεmX mAbs in various individuals, it is imperative to investigate the existence and the extent of genetic variation in CεmX in a sizable population. In this study, we analyzed the sequence of CεmX in 120 normal subjects and 200 asthmatic patients who reside in Taiwan.

Methods

Blood sample preparation

A group of 120 healthy volunteers (67 males and 53 females; age range: l6-52 years; average age: 23.4± 9.5 years), who had no history of allergy and whose routine pulmonary function tests revealed normal results, and of 200 patients (113 males and 87 females; age range: 7-68 years; average age: 25.8±15.2 years) with bronchial asthma were enrolled in this study. These patients had all tested positive for at least one of an array of local allergens using a radioimmunoassay test, and showed pulmonary dysfunctions such as chronic cough, wheezing dyspnea, chest tightness, and depressed FEV1 and FEV1/FVC (less than 80% of predicted values). Blood samples were

obtained from normal subjects and patients at China Medical University Hospital in Taichung City, located in the central west coast of Taiwan. The study was approved by the Institutional Review Board at China Medical University Hospital and performed according to the tenets of the Declaration of Helsinki for research involving human subjects. Informed consent was obtained from each individual enrolled in this study.

Determination of CεmX and migis-ε sequence

Genomic DNA was purified from whole blood using a MagNA Pure LC system (Roche Applied Science, Indianapolis, IN, USA). To avoid amplifying the membrane exon (me.p-me.1) from the ε pseudogene IGHEP1, the isolation of a segment containing CεmX-migis-ε from the functional IGHE gene was performed in two polymerase chain reaction (PCR) steps. The primers p1 (5′-CTCGTCTCT GGGTACACC-3′) corresponding to a segment in CH2 exon which is not present in IGHEP1, and p2 (5′-GCT GAGAGGAACCGCTGC-3′) corresponding to a segment in me.2 exon which is not present in IGHEP2, were used for the first round of PCR (Fig. 1a). PCR was carried out in a PX2 Thermal Cycler (Thermo Electron Corp., Milford, MA, USA) using a PCR kit (Advantage 2 Polymerase Mix, Clontech Laboratories, Palo Alto, CA, USA). Thirty nanograms of genomic DNA were used in a 50-μl reaction mixture. After an initial denaturation step at 95°C for 1 min, 20 reaction cycles (95°C for 30 s and 68°C for 3 min) were performed (the first PCR). The first PCR product was diluted 100-fold with ddH_2O, and 1 μl of the diluted PCR product was subjected to a second-round (nested) PCR, using p3 (5′-CGGGGCGCAGGGACGA GAG-3′) and p4 (5′-CACGCTGAGCAGGAAGAGT-3′) primers, which respectively correspond to a segment located in the intron upstream of the me.p exon and a segment in the me.1 exon of the IGHE gene (Fig. 1a). After an initial denaturation step at 95°C for 1 min, 30 reaction cycles (95°C for 30 s and 68°C for 1 min) were performed (the second PCR). The resultant PCR products were directly sequenced.

Measurement of serum IgE levels

Serum concentrations of IgE were measured using a commercial kit (Bethyl Laboratories, Montgomery, TX, USA) according to the manufacturer's protocols. The lower limit of detection in this assay is 15.6 ng/ml.

Flow cytometric analysis of cells expressing CεmX variants

293T cells, a human embryonic kidney cell line stably expressing the simian virus 40 large T antigen (ATCC, Rockville, MD, USA), were transfected with recombinant gene encoding mIgE.Fc_L(16V) or mIgE.Fc_L(16L), which contain sequence encoding the CH2 domain through the cytoplasmic end of mε_L(16V) or mε_L(16L) chains, using 25-kDa linear polyethylenimine as an auxiliary agent (Kirschner et al. 2006). The transfected cells, which expressed mIgE.Fc_L(16V) or mIgE.Fc_L(16L), were then incubated with 10 μg/ml mouse anti-IgE mAb (5H2, AbD Serotec, Oxford, UK) or mouse anti-CεmX mAb (a20, 4B12, or 26H2), followed by FITC-labeled rabbit $F(ab')_2$ specific for mouse IgG (AbD Serotec). The stained cells were analyzed on a FACSCanto II flow cytometer (BD Biosciences, San Jose, CA, USA).

ELISA measuring reactivity of anti-CεmX mAb with synthetic peptides

Two peptides representing the N-terminal 17-a.a. segment of CεmX variants (with 16V and 16L) were synthesized at the Genomics Research Center, Academia Sinica (Taipei, Taiwan). Each peptide was reconstituted with phosphate buffered saline (PBS) to a concentration of 10 mg/ml and diluted with $NaHCO_3$ buffer (0.1 M, pH 9.6) to 10 μg/ml for use in coating wells of 96-well MaxiSorp plates (NUNC, Roskilde, Denmark). After the wells were incubated with the peptide overnight at 4°C, blocked with PBS containing bovine serum albumin, and washed, the wells were incubated with the 4B12 mAb at different concentrations for 1 h and its binding to the wells was measured by horse radish peroxidase-conjugated goat anti-mouse IgG antibody (Chemicon, Billerica, MA, USA) using SureBlue 3,3′,5,5′- tetramethylbenzidine (KPL, Inc., Gaithersburg, MD, USA) as a colorimetric substrate.

Results

Sequencing of CεmX and migis-ε regions

To examine possible genetic variations in the CεmX and migis-ε regions of mε, a 201 bp segment of genomic DNA encompassing me.p (156 bp) and me.1 (45 bp) was examined in 320 individuals, who were all of Chinese ethnicity residing in Taiwan. Among the individuals enrolled in this study, 120 were healthy and 200 were patients with asthma who had tested positive to at least one local allergen.

A large gene segment of 3,231 bp, which extends from CH2 to me.2 of IGHE, was first amplified by p1 and p2 primers as shown in Fig. 1a. A subsequent, nested PCR was then performed using p3 and p4 primers to amplify a segment of 331 bp harboring the 201 bp CεmX and migis-ε segments, with flanking sequences on both ends, again

shown in Fig. 1a. In the present study, the nucleotide positions are simply numbered from 1 to 201, and amino acid positions 1-67, along the contiguous CεmX and migis-ε segments (Fig. 2a). From a total of 320 direct DNA sequencing determinations, three distinct sequences were identified. One was as previously described (GeneBank accession no. S71428), while the other two have not been reported before. One of the two new sequences has a single-nucleotide difference (G/T) at nucleotide #46, which results in a change from Val to Leu at amino acid residue #16. The other genetic variant has a single-nucleotide polymorphism (SNP; A/G) at nucleotide #93, which does not result in an amino acid change (still Gly). No polymorphism was found in the migis-ε region. The three "alleles" of the CεmX region are here referred to as "46G93A", "46T93A", and "46G93G"—the first is previously known and the latter two are newly discovered.

Distribution of CεmX genetic variants in the Taiwanese population

Of the 640 genomic sequences of the 320 subjects studied, there are 614 copies of 46G (95.9%) and 26 copies of 46T (4.1%); 344 copies of 93A (53.8%) and 296 copies of 93G (46.2%; see Table 1). Among the three allelic forms, there are 221 copies of 46G93G (34.5%), 26 copies of 46T93A (4.1%), and 293 copies of 46G93G (45.8%; Table 1). The most frequently found allelic form is not the previously known 46G93A, but the newly found 46G93G. There was no copy of 46T93G found in the present sample set.

The six diploid combinations and their respective distribution among the 320 subjects are shown in Table 2. At the protein structure level, there are three variants, 16V/16V (92.5%), 16V/16L (6.9%), and 16L/16L (0.6%). These data indicate that 99.4% of people have 16V and 7.8% have 16L.

Preliminary assessment of possible correlation of CεmX polymorphism with asthma

The extent of any correlation between SNPs and genotypes of CεmX of subjects and their serum IgE levels was analyzed. The data from 193 asthmatic patients (Table 3) show that 46G/T and 93A/G polymorphisms were not associated with serum IgE levels. The rationale for including both healthy subjects and patients with allergic asthma in the present study was to address whether or not genetic variations in CεmX, if found, would correlate with IgE-mediated diseases. Statistical analyses comparing the proportions of SNPs and genotypes in CεmX between healthy subjects and asthmatic patients were not conclusive and have not been presented here.

Anti-CεmX mAbs bind to mIgE.Fc_L(16V) and mIgE.Fc_L(16L) equally well

Our group has generated mouse anti-CεmX mAbs, including a20 (Chen et al. 2002) and 26H2 and 4B12 (Chen et al. 2010), which bind to the C-terminal, middle, and N-terminal parts of CεmX, respectively. The binding epitope for a20 is RADWPGPP (#45-52), for 26H2 is GQQQGLPRAAG (#21-31), and for 4B12 is GLAGGS AQSQRAPDRV (#1-16). To determine the reactivity of those mAbs to variants of mIgE, their binding to 293T cells

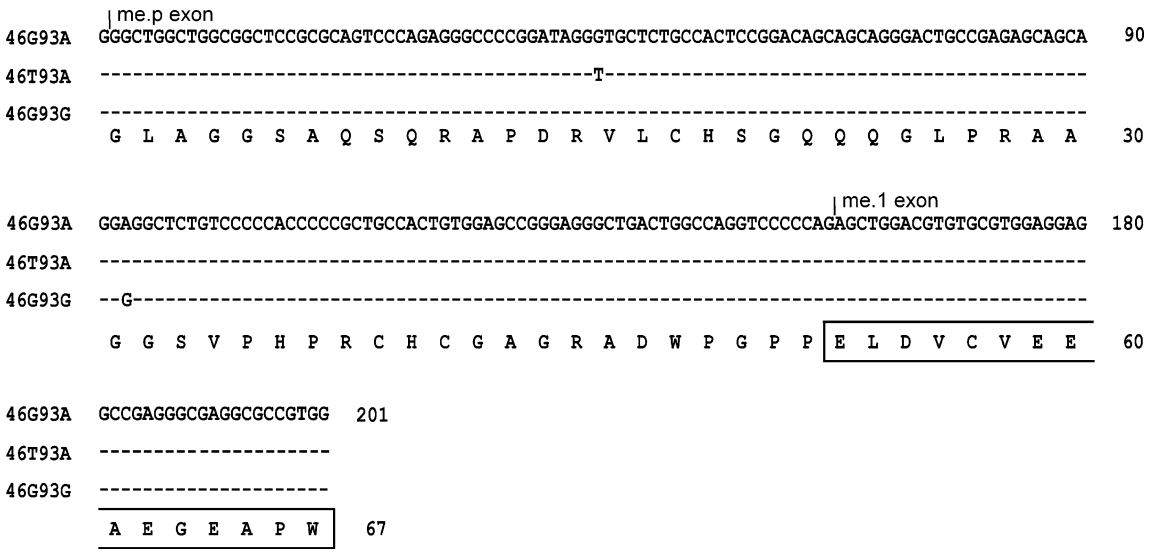

Fig. 2 The three allelic forms of CεmX. In the 320 subjects studied in Taiwan, in addition to 46G93A (GeneBank accession no. S71428), two new allelic forms 46T93A (GeneBank accession no. GU208817) and 46G93G (GU208818) were found. No 46T93G was found. No SNP was identified in the migis-ε segment (*boxed*). The nucleotide positions are numbered from 1 to 156 along the length of the CεmX segment

Table 1 The proportions of different SNPs and of allelic forms of CεmX among the studied Taiwanese population

Polymorphism		Healthy subjects n=120 Number (%)	Asthmatic patients n=200	Total subjects n=320
SNP				
#46	G	236 (98.3)	378 (94.5)	614 (95.9)
	T	4 (1.7)	22 (5.5)	26 (4.1)
#93	A	118 (49.2)	226 (56.5)	344 (53.8)
	G	122 (50.8)	174 (43.5)	296 (46.2)
Allelic form				
46G93A		175 (43.8)	118 (49.2)	293 (45.8)
46T93A		22 (5.5)	4 (1.7)	26 (4.1)
46G93G		203 (50.8)	118 (49.2)	321 (34.5)

transiently expressing mIgE.Fc_L(16V) or mIgE.Fc_L(16L) was examined. All three mAbs bound to mIgE.Fc_L(16V) and mIgE.Fc_L(16L) equally well (Fig. 3a). The mAb 4B12 bound to GLAGGSAQSQRAPDRVL and GLAGGS AQSQRAPDRLL peptides equally well over a large concentration range in an ELISA (Fig. 3b).

Discussion

Since the discovery of CεmX was reported in 1992, there has not been a single report of any genetic variation in the 156 bp CεmX. Since anti-CεmX mAbs have now been proposed as a therapeutic agent to target mIgE-expressing B cells (Chang et al. 2007; Chen et al. 2002), this study set out to confirm the lack of genetic variation in CεmX. It was somewhat unexpected that in the present study, two SNPs in the CεmX region of IGHE, namely 46G/T and 93A/G, were identified among 320 individuals residing in Taiwan. Three allelic forms, 46G93A, 46T93A, and 46G93G, and six diploid combinations, 46G93A/46G93A, 46T93A/46T93A, 46G93G/46G93G, 46G93A/46T93A, 46G93G/46G93A, and 46G93G/46T93A, were found to exist in this examined sample set. All 24 individuals carrying 46T also bear 93A. The absence of a 46T93G allelic form may possibly be due to either (1) the 46G/T or 93A/G change occurred relatively recently or (2) the sample size is not sufficiently large to observe a possibly extant 46T93G at low frequency.

In the latest human genome assembly (version GRCh37), the CεmX region in the functional IGHE and in the pseudogene IGHEP1 is identical, except that IGHE has 93A and IGHEP1 has 93G. Now that we have found IGHE itself also has an allelic 93G form, it is possible that after the ε-α duplication (Hisajima et al. 1983; Max et al. 1982), subsequent deletion in one ε gene created the pseudogene IGHEP1 and a single-nucleotide mutation from 93G to 93A occurred in IGHE. Analogously, in the existing database, both IGHE and IGHEP1 have 46G. It is possible that the variation of 46G/T in IGHE (with 93A) was due to a change

Table 2 The proportions of different genotypes of CεmX among the studied Taiwanese population

Polymorphism	Healthy subjects n=120 Number (%)	Asthmatic patients n=200	Total subjects n=320
Genotype			
46G/46G	116 (96.7)	180 (90.0)	296 (92.5)
46G/46T	4 (3.3)	18 (9.0)	22 (6.9)
46T/46T	0 (0)	2 (1.0)	2 (0.6)
93A/93A	32 (26.7)	84 (42.0)	116 (36.3)
93A/93G	54 (45.0)	58 (29.0)	112 (35.0)
93G/93G	34 (28.3)	58 (29.0)	92 (28.7)
46G93A/46G93A	71 (35.5)	32 (26.7)	103 (32.2)
46T93A/46T93A	2 (1.0)	0 (0)	2 (0.6)
46G93G/46G93G	58 (29.0)	32 (26.7)	90 (28.1)
46G93A/46T93A	10 (5.0)	2 (1.7)	12 (3.8)
46G93G/46G93A	51 (25.5)	52 (43.3)	103 (32.2)
46G93G/46T93A	8 (4.0)	2 (1.7)	10 (3.1)

Table 3 Serum IgE levels in subjects with different CεmX genotypes

Genotypes	Asthma patients Numbers (%)	IgE concentration Mean±SD; ng/ml
46G/46G	180 (93.3)	440±752
46G/46T	11 (5.7)	190±273
46T/46T	2 (1.0)	1,040±373
93A/93A	79 (41.0)	443±765
93A/93G	57 (29.5)	342±400
93G/93G	57 (29.5)	506±928

from G to T and not T to G. The relative proportion of 46G to 46T being 95.9-4.1% supports such a notion. The absence of 46T93G is also consistent with such a suggestion.

The duplication of the ε-α cluster resulted in two functional α genes. As time elapsed, a few mutations occurred that distinguished the IGHA1 and IGHA2 loci. One of the mutations in IGHA1 was the creation of an alternative splicing acceptor site. Thus, for IGHA1, a "short" (conventional) form and a "long" form of membrane-bound α1 chains are expressed, with the latter containing an extra six amino acid residues at the N-terminus of the transmembrane peptide segment (Yu et al. 1990). The IGHA2 locus merely produces the short form of membrane-bound α2 (Leduc et al. 1997; Yu et al. 1990). Furthermore, our group previously reported that a new

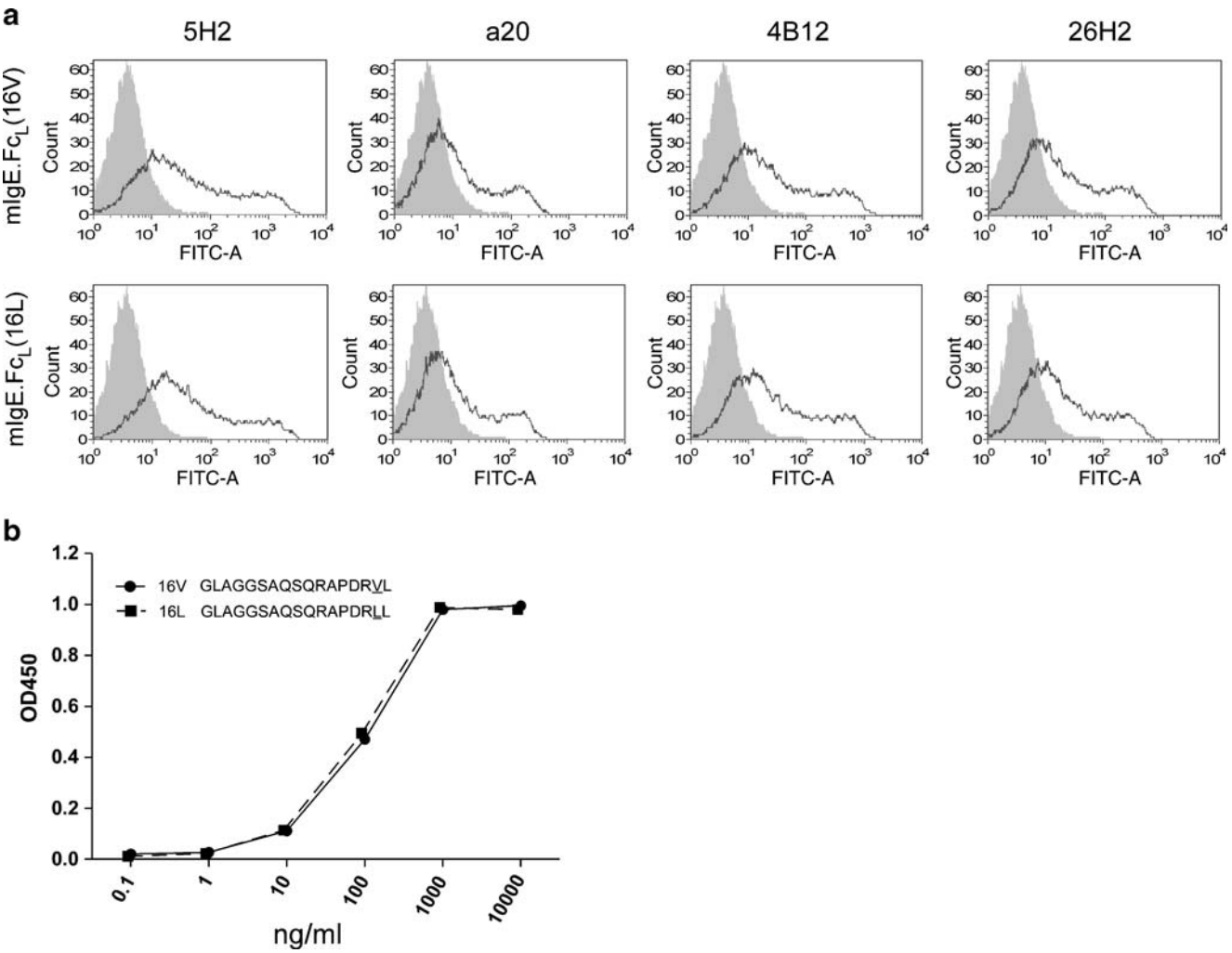

Fig. 3 Binding of anti-CεmX mAbs to variants of mIgE. **a** Flow cytometric analysis of anti-CεmX mAbs binding to variants of mIgE. Fc_L on the cell surface. 293T cells transfected with cDNAs encoding mIgE.Fc_L(16V) or mIgE.Fc_L(16L) were incubated with non-immune mouse IgG (*filled histogram*), anti-IgE mAb (5H2), or anti-CεmX mAbs (a20, 4B12, or 26H2), followed by staining with FITC-labeled rabbit F(ab′)$_2$ specific for mouse IgG. **b** ELISA analysis of 4B12 mAb binding to synthetic peptides containing its epitope. Each well was coated with 10 μg/ml of GLAGGSAQSQRAPDRVL or GLAGG SAQSQRAPDRLL peptides. The secondary antibody was peroxidase-conjugated goat anti-mouse IgG. Data are averages of triplicate measurements

allelic form of IGHA1, mα1(456C), in addition to the previously known form, mα1(456S), is present in the Taiwanese population (Hung et al. 2008).

The genetic variation at 46G/T in CεmX causes a Val to Leu change at amino acid residue #16. While Val and Leu are generally considered to be branched-chained amino acids with similar chemical properties, such a change has been found in some cases to cause significant changes in the properties of the resultant proteins. For example, a Val to Leu change at residue #3 of the A chain of insulin decreases the hormone's binding affinity to its receptor and is a cause of diabetes (Nanjo et al. 1986; Sakura et al. 1986; Wan et al. 2005). A similar change at residue #34 of factor XIII enhances the factor's sensitivity toward thrombin activation, a cause of thrombosis (Catto et al. 1998; Corral et al. 2000), and at residue #190 of hemoglobin causes the protein to bind oxygen more tightly, leading to erythrocytosis and thalassemia (Jones et al. 1990; Ropero et al. 2000). CεmX is implicated in certain functions as $mIgE_L$ and $mIgE_S$ have been reported to associate with Igα differently and to transduce the signals originating from IgE-BCR differently (Batista et al. 1996). When the function of CεmX is more clearly unraveled, the possible effect of 16V/L variation can then be addressed.

The clinical efficacy of omalizumab in patients with allergic asthma and other allergic diseases has validated the therapeutic approach of down-regulating IgE for treating IgE-mediated allergic diseases. An alternative to omalizumab, which binds to free IgE and mIgE-expressing B cells, is an anti-CεmX antibody which targets IgE-expressing B cells without binding to free IgE. An anti-CεmX mAb is not consumed by free IgE and can target mIgE-expressing B cells in patients with very high free IgE levels. The mAb 4B12 could lyse $mIgE.Fc_L$-expressing Ramos cells through BCR-mediated apoptosis and antibody-dependent cellular cytotoxicity. In a culture where the synthesis of IgE by PBMCs from patients with atopic dermatitis was induced by anti-CD40 and IL-4, the mAb 4B12 could inhibit such a process (Chen et al. 2010). We propose that 4B12 can potentially be used to target IgE-committed B cells to modulate IgE production in allergic patients. While the parental mAb 4B12 was originally isolated in a mouse immunized with a recombinant protein containing CεmX (16V), 4B12 can bind indistinguishably well to GLAGGSAQSQRAPDRVL or GLAGGSAQSQRAPDRLL and to 293T cells transiently expressing $mIgE.Fc_L$(16V) or $mIgE.Fc_L$(16L). The results here indicate that if 4B12 is chosen as a therapeutic agent for targeting $mIgE^+$ B cells, the newly discovered polymorphisms in CεmX in the Taiwanese population would not affect the general applicability of mAb 4B12 in this population.

The studies undertaken by our group on the genetic variation of α and ε immunoglobulins in the Taiwanese population have identified several new alleles, some leading to amino acid variations, in both genes. Whether these genetic variations in the CεmX domain bear any medical relevance remains to be studied further. When the humanized anti-CεmX antibody, 4B12, is advanced to preclinical developmental stage, possible genetic variations in the CεmX domain in various ethnic groups will be investigated.

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