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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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Three-dimensional structure of the wheat β-amylase Tri a 17, a clinically relevant food allergen

To the Editor,

Wheat is one of the most important staple foods worldwide and has been recognized as a potent food allergen source. Allergies are on the rise in western countries and the prevalence of food allergy has reached approximately 7% in children in the United States,[1] making improved diagnostics an important goal. Not all known potential allergens of wheat have been characterized so far and the prevalence of true wheat allergy is difficult to determine due to IgE cross-reactivity with grass pollen allergens.[2]

The wheat β-amylase (Tri a 17) has been found to bind IgE of wheat allergic patients,[3] but its structure and allergenic activity have not been studied. To evaluate its possible clinical relevance and to shed some light on its biochemical properties, we elucidated the three-dimensional structure, measured the enzymatic activity, IgE-binding capacity, and allergenic activity of the recombinant enzyme.

The β-amylases of crop plants like wheat, soy, or barley have been under investigation for decades and the presence of two general forms has been described. One protein isoform found in all parts of the plant, termed ubiquitous β-amylase, and one variant specific to the endosperm, featuring an elongated C-terminal tail region are known. To date, only the ubiquitous form of wheat β-amylase has been sequenced.[4]

We screened a wheat seed cDNA library with serum IgE antibodies from patients suffering from wheat-induced food allergy and identified an IgE-reactive cDNA clone which was homologous to the barley endosperm β-amylase at its C-terminus and to the sequence of wheat ubiquitous β-amylase at its N-terminus.

We then expressed a recombinant protein consisting of the ubiquitous wheat β-amylase sequence fused with the recovered IgE-reactive C-terminus (named Tri a 17_clone) and the unaltered ubiquitous β-amylase (termed Tri a 17_inactive) both as inclusion bodies in *Escherichia coli*. A refolding attempt yielded soluble, yet misfolded and aggregated protein. However, we were able to optimize the expression conditions to obtain natively folded amylase (named Tri a 17_active) (Figure S1 and S2 and the Methods section in this article's Online Repository).

The far-UV CD spectra of the inactive proteins indicate the presence of β-sheets and random coil signal, while Tri a 17_active exhibits the high α-helical content of a TIM-barrel (Figure 1B).

Purified Tri a 17_active crystallized readily and the structure was solved by X-ray crystallography to a resolution of 2 Å (PDB: 6GER). The structure superimposes well with the previously published structure of barley β-amylase (rmsd = 0.62 Å when 442 of 486 Cα atoms are aligned with the PDB structure 2XFF[5]), which was used as the template for molecular replacement.

The crystal structure of Tri a 17_active shows the same $(\beta/\alpha)_8$-barrel architecture found for other plant and bacterial β-amylases (Figure 1A).

Figure 1C shows the variation in the relative enzymatic activity of the β-amylase with respect to pH. The enzyme shows a tolerance

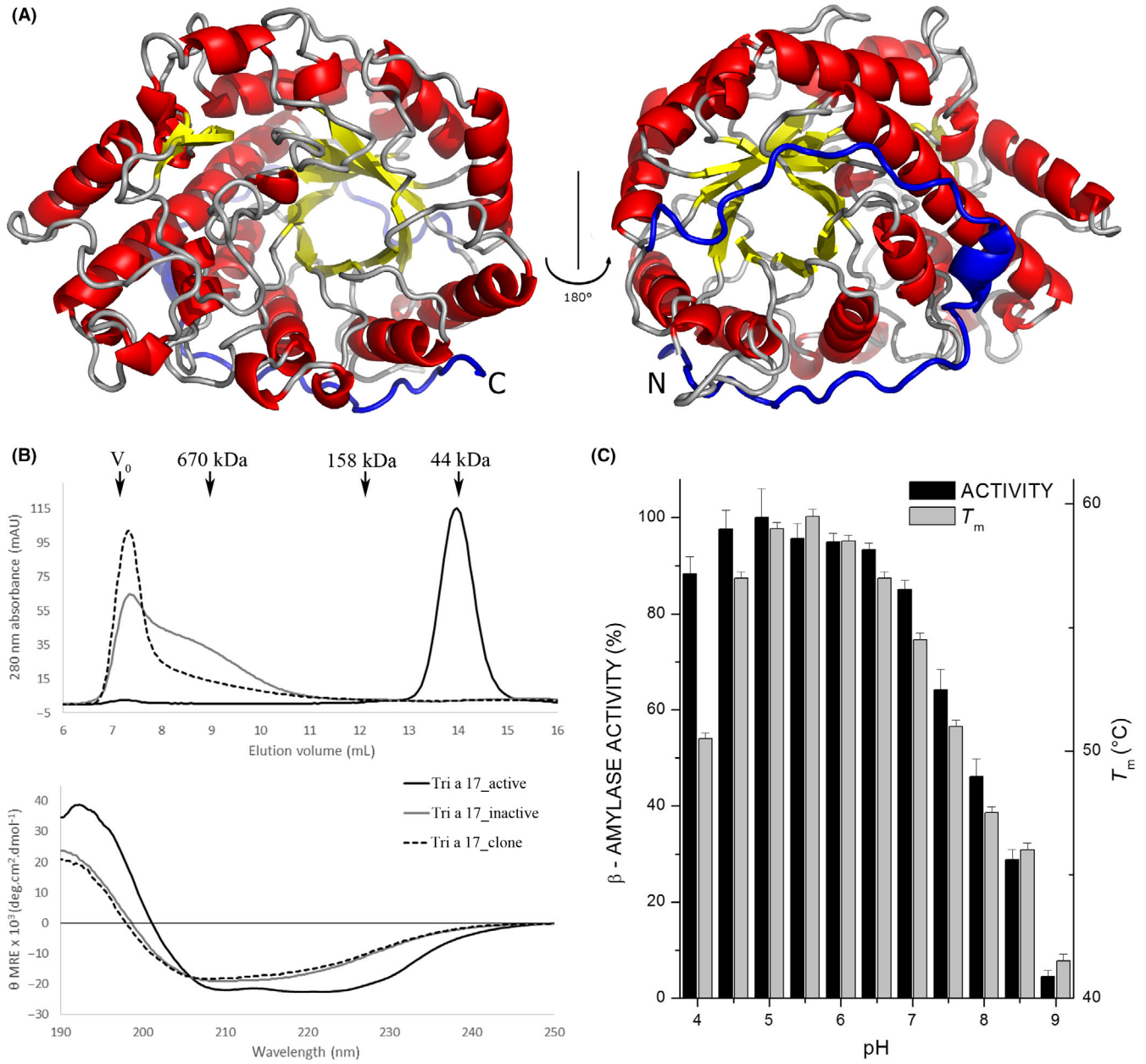

FIGURE 1 A, Cartoon model of Tri a 17 showing the $(\beta/\alpha)_8$-barrel architecture with its C-terminal portion, which corresponds to the IgE-reactive peptide, in blue. B, Size exclusion chromatography traces of the recombinant amylases, marker protein elution volumes, and void volume are shown with arrows (top). Circular dichroism analysis of recombinant β-amylases. The spectra are expressed as mean residue ellipticities (θ-MRE) (*y*-axis) at given wavelengths (*x*-axis) (bottom). C, Percent maximum activity of Tri a 17_active as a function of pH (black). Tri a 17_active melting temperatures (T_m) against pH as measured by DSF (gray). Data are averages of three independent measurements with error bars showing the standard deviation

for low pH, showing maximum activity at pH 5 and retaining over 80% of its maximum activity even at pH 4. However, at higher pH values, a marked decrease in activity is seen, with more than 50% activity lost at pH 8. In general, Tri a 17 is highly active in a wide range of acidic pH conditions (4.0-7.0), similar to other β-amylases, such as the major β-amylases of barley.[6]

Figure 1C also shows the melting temperature of Tri a 17_active at different pH values, as measured by differential scanning fluorimetry. The protein is most stable in slightly acidic conditions. A maximum in melting temperature is seen at pH 5.5 with 59°C, with a tail off to lower values as pH increases and a sharp drop in stability at pH 4.

The IgE reactivity of the three forms of Tri a 17 was assessed in non-denaturing RAST-based IgE dot blot experiments with sera from 17 wheat food allergic patients. Tri a 17_clone and Tri a 17_inactive were recognized by 24% (4 of 17) of wheat food allergic patients with varying intensities, whereas the folded enzyme (Tri a 17_active) was recognized by 41% (7 of 17) of wheat food allergic patients, indicating the presence of conformational IgE epitopes as well as linear epitopes. Since the enzymatic activity depends on the correct

fold, there is an apparent correlation between enzymatic and allergenic activity of the β-amylase. IgE reactivity was specific to patients suffering from wheat food allergy. Non-allergic individuals (patients 18 and 19), grass pollen allergic patients (patients 20 and 21), and baker's asthma patients (patients 22 and 23) did not exhibit IgE reactivity to either form of beta amylase (Figure 2A).

Interestingly, 8 out of 17 wheat food allergic patients (47%) had a history of wheat-induced anaphylaxis. Of those, six were among the seven β-amylase positive patients. This correlation between anaphylaxis and Tri a 17 IgE recognition is statistically significant ($P = 0.015$). The relative risk of developing wheat-induced anaphylaxis, estimated by logistic regression, indicates a 24-fold higher

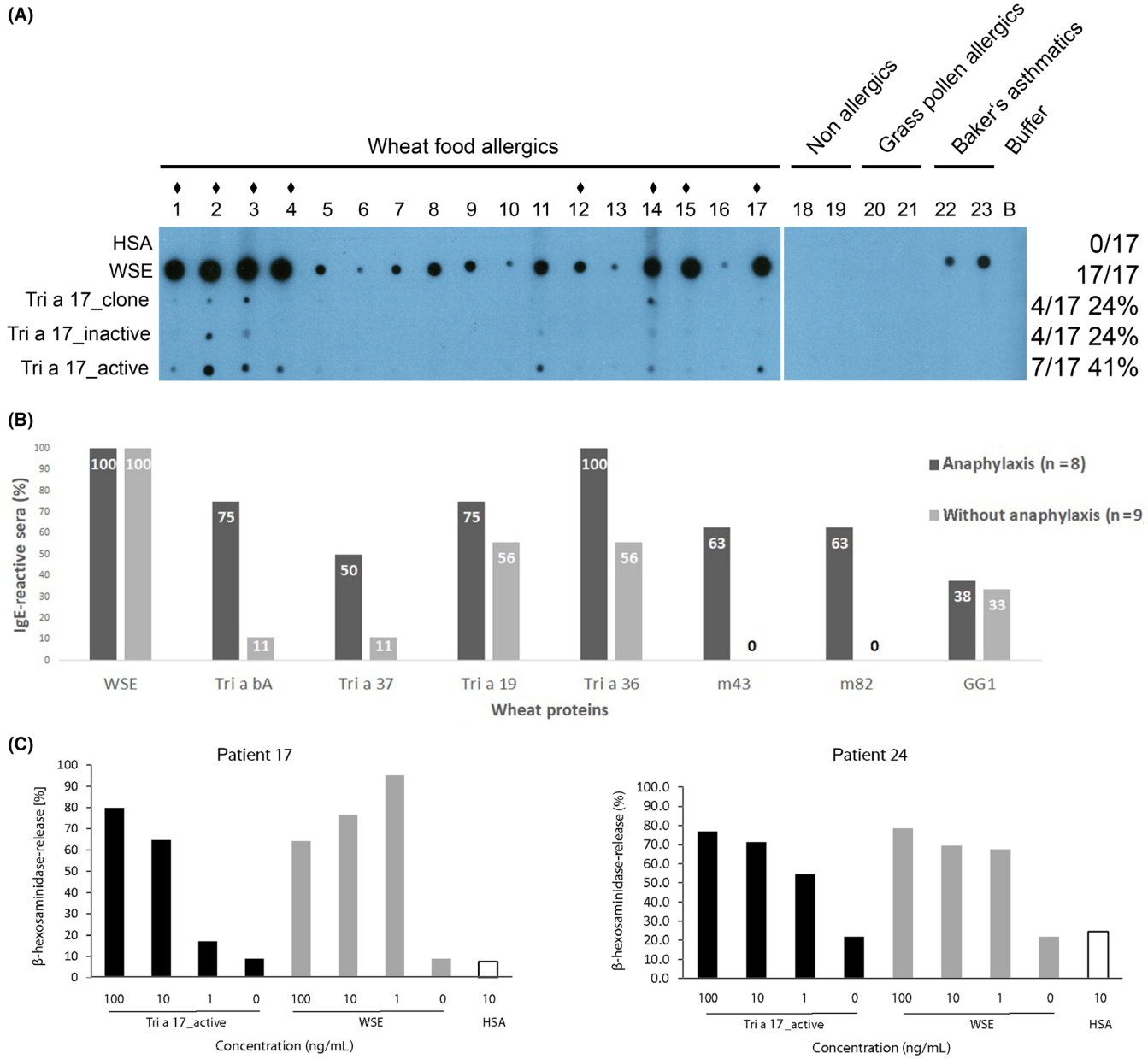

FIGURE 2 A, IgE reactivity of wheat β-amylases (Tri a 17_clone, Tri a 17_inactive and Tri a 17_active). Nitrocellulose-dotted human serum albumin (HSA), wheat seed extract (WSE), Tri a 17_clone and Tri a 17, in its inactive and active form, were tested with sera from wheat food allergic patients (1-17). For control purposes, sera from non-allergic individuals (18 and 19), grass pollen allergic patients (20 and 21) baker's asthma patients (22 and 23), or buffer alone (B) were included. Patients with a history of wheat-induced anaphylaxis are indicated by ♦ above their number. B, IgE reactivity of wheat food allergic patients with and without anaphylaxis. IgE-binding prevalences (*y*-axis: percentage of IgE-reactive sera) to wheat seed extract and wheat proteins (Tri a 17, Tri a 37, Tri a 19, Tri a 36, m43, m82, GG1) for patients with (black bars) and without anaphylaxis (gray bars). C, Allergenic activity of recombinant β-amylase Tri a 17_active and wheat seed extract. RBL cells were loaded with serum IgE from Tri a 17_active-reactive patients and incubated with increasing concentrations of Tri a 17_active (black) or WSE (gray) (100, 10, 1 ng/mL), buffer alone (0 ng/mL) or HSA (white) (10 ng/mL). β-Hexosaminidase releases are displayed as percentages of total β-hexosaminidase release on the *y*-axes

probability for β-amylase-reactive patients (Methods, Online Repository). Figure 2B shows the IgE recognition frequencies for the patients with and without anaphylaxis for β-amylase (Tri a 17) and other wheat allergens. High molecular weight glutenins (m43, m82,[7] $P = 0.009$), low molecular weight glutenin (Tri a 36,[8] $P = 0.08$), and alpha purothionin (Tri a 37,[9] $P = 0.13$) also discriminate between patients with and without anaphylaxis, to varying degrees. However, omega 5 gliadin (Tri a 19, $P = 0.62$), gamma gliadin (GG1[10]), and wheat seed extract do not discriminate between patients with and without anaphylaxis.

Tri a 17_active was able to induce effector cell degranulation in basophil degranulation assays. Human FcεRI-expressing RBL cells were passively sensitized with sera from wheat food allergic patients which showed IgE reactivity to Tri a 17 (Figure 2A, Figure S3). Subsequent incubation with increasing concentrations of Tri a 17_active or wheat seed extract (WSE) showed release for both wheat food allergic patients tested (Figure 2B).

Within a barley kernel, β-amylases can reach 1%-2% of the total protein in the starchy endosperm.[6] Assuming similarly high expression in wheat, amylase concentrations that showed effector cell release (1 μg/L) are likely to be found in all flour-containing foodstuffs.

The multiple sequence alignment of β-amylase showed that highly homologous proteins occur in various plant species (Figure S2 in this article's Online Repository). Cross-reactive antigens were detected in rye, maize, oat, spelt, barley, soy, sunflower, and rice using rabbit sera.

The protein was detected in all processed cereal products (ie, brown bread, rye bread, and rolls) including gluten-free bread and even after baking, showing a remarkable persistence which may explain why it can induce severe reactions (Figure S4 in this article's Online Repository).

Based on our findings, wheat beta amylase has been given the official name "Tri a 17" by the WHO/IUIS allergen nomenclature subcommittee.

In summary, wheat β-amylase can be classified as a class I food allergen that sensitizes via the gastrointestinal tract.

Wheat β-amylase seems to be associated with severe allergic reactions upon wheat ingestion in sensitized patients and should be included in the panel of potential diagnostic marker molecules for severe wheat food allergy.

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CONFLICT OF INTEREST

Rudolf Valenta has received research grants from Biomay, Vienna, Austria and Viravaxx, Vienna, Austria and serves as a consultant for both companies. The other authors declare no conflicts of interest.

ORCID

Gerhard Hofer https://orcid.org/0000-0002-9248-6989
Michael K. Bogdos https://orcid.org/0000-0003-1826-2706
Ryosuke Nakamura https://orcid.org/0000-0002-7756-3798
Nikolaos Papadopoulos https://orcid.org/0000-0002-4448-3468
Rudolf Valenta https://orcid.org/0000-0001-5944-3365
Walter Keller https://orcid.org/0000-0002-2261-958X

Gerhard Hofer[1]
Sandra Wieser[2]
Michael K. Bogdos[1]
Pia Gattinger[2]
Ryosuke Nakamura[3]
Motohiro Ebisawa[4]
Mika Mäkelä[5]
Nikolaos Papadopoulos[6]
Rudolf Valenta[2,7,8]
Walter Keller[1]

[1]*Institute of Molecular Biosciences, BioTechMed Graz, University of Graz, Graz, Austria*
[2]*Division of Immunopathology, Department of Pathophysiology and Allergy Research, Center for Pathophysiology, Infectiology and Immunology, Medical University of Vienna, Vienna, Austria*
[3]*Division of Medicinal Safety Science, National Institute of Health Sciences, Kanagawa, Japan*
[4]*Clinical Research Center for Allergology and Rheumatology, National Hospital Organization, Sagamihara National Hospital, Kanagawa, Japan*
[5]*Department of Allergology, Skin and Allergy Hospital, Helsinki University Central Hospital, Helsinki, Finland*
[6]*Allergy Department, 2nd Pediatric Clinic, University of Athens, Athens, Greece*
[7]*NRC Institute of Immunology FMBA of Russia, Moscow, Russia*
[8]*Laboratory for Immunopathology, Department of Clinical Immunology and Allergy, Sechenov First Moscow State Medical University, Moscow, Russia*

Correspondence
Walter Keller, Institute of Molecular Biosciences, BioTechMed Graz, University of Graz, Graz, Austria.
Email: walter.keller@uni-graz.at
Wieser and Hofer contributed equally to the work.

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Genotype-phenotype correlations in Brazilian patients with hereditary angioedema due to C1 inhibitor deficiency

To the Editor,

Hereditary angioedema (HAE) is a rare disease with autosomal dominant inheritance that affects 1 in every 50 000 individuals.[1] Patients with HAE present recurrent episodes of edema of subcutaneous tissue and submucosa that mainly affects the skin, gastrointestinal tract, and upper airways. In most cases, the disease results from the deficiency of C1 inhibitor (C1-INH) owing to mutations in *SERPING1*, the gene encoding C1-INH protein. The decrease in C1-INH activity leads to uncontrolled activation of the kallikrein-kinin system and increased formation of bradykinin, resulting in angioedema. HAE with normal C1-INH has also been characterized.[2] Diagnosis of C1-INH-HAE is based on clinical symptoms, positive family history, low levels and/or functional activity of C1-INH, and decreased C4.[1] Whether genetic analysis should be performed in routine clinical practice is yet debated. Variability in clinical presentation of HAE has prompted researchers to look for novel biomarkers. Kaplan and Maas have recently discussed the role of potential biomarkers, including blood levels of bradykinin or the pentapeptide Arg–Pro–Pro–Gly–Phe derived from bradykinin degradation and cleaved high-molecular-weight kininogen, in assessing HAE severity and response to therapeutic agents.[3] The type of mutation in *SERPING1* could account for clinical phenotypes. Patients with missense mutations have been shown to present symptoms at later ages, milder clinical course, and lesser need for prophylactic medications than those with mutations that cause more profound changes in the molecule.[4,5] Grouping patients with missense mutations in *SERPING1* affecting Arg466 at the reactive center with those carrying mutations leading to more significant changes in C1-INH molecule revealed association with more severe disease.[6,7] In the present study, we aimed to identify and characterize mutations in *SERPING1* in Brazilian patients with C1-INH-HAE and investigate the impact of the type of mutation on clinical features of the disease.

Sixty patients with C1-INH-HAE from 16 distinct families were characterized based on mutations in *SERPING1*. Diagnosis was established according to consensus criteria (Data S1).[1] One of the families (Family 7) has been previously reported by our group; we provide follow-up information, including five new members with HAE-1.[8] Patients were divided into the following groups: group 1 comprising patients with deletion, insertion, duplication, or nonsense mutation and mutations affecting Arg466 at the reactive center (n = 48); and group 2 comprising patients with missense mutations, with the exception of mutations at Arg466 (n = 12). The rationale for dividing patients into these groups was the fact that mutations causing more profound alterations in the structure of the protein could lead to more severe disease, as previously reported.[6,7]

Genomic DNA was extracted from whole blood or oral mucosa material using the DNA Wizard Genomic DNA Purification Kit (Promega, Madison, WI). PCR was conducted using *SERPING1* primers (Data S2, Table S1), and DNA sequences were analyzed using the SeqMan software™ (Lasergene; DNA Star, Inc., Madison, WI). Sequence variations were identified by comparison with GenBank accession number NM_000062.2, X54486. Protein effect was defined using the mature protein sequence, including signal peptide. Criteria of the American College of Medical Genetics and Genomics (ACMG) were applied for classifying sequence variants. Predicted functional analysis of missense mutations was performed using the bioinformatics tools SIFT (http://sift.jcvi.org/www/SIFT_enst_submit.html), PolyPhen2 (http://genetics.bwh.harvard.edu/pph2/), and MutationTaster,