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A Synonymous Polymorphism of *APCDD1* Affects Translation Efficacy and is Associated with Androgenic Alopecia

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Abstract: The *APCDD1* (*adenomatosis polyposis coli down-regulated 1*) gene is an inhibitor of the Wnt signaling pathway, and a rare mutation of this gene has been associated with hereditary hypotrichosis simplex. In this study, the authors aimed to investigate whether common *APCDD1* gene polymorphisms contribute to the development of androgenic alopecia. Patients (n = 210) with androgenic alopecia and 98 controls were investigated. SNPs (Single nucleotide polymorphisms) in the coding region of the gene were sequenced. A significant difference in genotype distribution was found for the c.1781C/T, p.L476L SNP (rs3185480) of the *APCDD1* gene. This SNP is located in exon 5 and is associated with a 3.5- and a 2.8-fold increase in risk for the development of androgenic alopecia for homozygote (CI 0.933-13.125; nominal regression P = 0.063) and heterozygote (CI 1.086-7.217; nominal regression P = 0.033) carriers, respectively. These data suggest that the rs3185480 polymorphism contributes to the development of androgenic alopecia. Protein expression experiments revealed that the polymorphism is associated with reduced APCDD1 protein abundance. This reduction is likely due to altered codon usage for leucine from a preferred codon (CTC) to a rare codon (CTT), which might influence translation efficiency and, thus, APCDD1 protein level.

Key words: *Adenomatosis polyposis coli down-regulated 1* gene, hereditary hypotrichosis simplex, androgenic alopecia, polymorphism, synonymous, translation efficacy, preferable codon.

1. Introduction

Adenomatosis polyposis coli down-regulated 1 (*APCDD1*) was initially isolated in a search for genes that were down-regulated after introduction of an adenovirus construct expressing the wild type *APC* gene into SW480 colon cancer cells [1]. APCDD1 is a membrane-bound glycoprotein, conserved throughout vertebrate evolution [1]. The *APCDD1* gene is abundantly expressed in follicular dermal papilla cells, hair matrix and hair shafts and is an inhibitor of the Wnt signaling pathway [2-4]. In addition to those associated with hair, APCDD1 is expressed widely in various cell types and, therefore, may regulate several biological processes controlled by Wnt signaling [2, 5].

A recurrent missense mutation (p.Leu9Arg) of this gene has been shown to be associated with the rare hair condition hereditary hypotrichosis simplex (OMIM 605389), which is an autosomal dominant form of nonsyndromic alopecia [3, 4]. The p.Leu9Arg mutation is located in the signal sequence of the APCDD1 protein and impairs its transport to the plasma membrane [3]. The hereditary hypotrichosis

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simplex associated with p.Leu9Arg-APCDD1 is characterized by normal hair at birth and a progressive hair loss starting in early childhood. Hair loss affects the scalp only or the scalp and axillary and pubic hair, but not beard hair [3, 4]. The p.Leu9Arg-APCDD1 associated hypotrichosis also involves follicle miniaturization, resulting in the development of thin and short hair, which is also a typical feature of androgenetic alopecia (AGA, OMIM 109200) [3, 4, 6].

AGA, the most common hair-loss disorder, is a multifactorial and polygenic disorder with several well-known acquired and inherited factors in its etiology. The disease occurs in both men and women and is characterized by hair loss following defined patterns [7, 8]. The patterns of hair loss for men are described by the Hamilton-Norwood scale and for women by the Ludwig scale [7]. Initial signs of AGA usually develop during teenage years and lead to progressive hair loss. Its frequency increases with age and affects up to 80% Caucasian men and 42% Caucasian women [9, 10]. AGA is characterized by the transformation from terminal to vellus hairs, shortening of the anagen phase, miniaturization of the hair follicles (and, thus, progressive thinning of the hair shafts) and progressive hair loss [10].

A recurrent missense mutation of the *APCDD1* gene results in the development of a rare hair disorder, hereditary hypotrichosis simplex. This observation led us to ask whether common SNPs of this gene contribute to the pathogenesis of AGA.

2. Patients and Methods

2.1 Patients

Three hundred and eight Hungarian individuals were enrolled into the study. Two hundred and ten of the enrolled individuals were AGA patients and 98 were healthy individuals. The severity of AGA was classified according to the Hamilton-Norwood scale for men and the Ludwig scale for women. The female to male ratio was 2:1 in both AGA and control groups. The average age was 45 ± 27 years for AGA patients and 43 ± 25 for controls. The average body weight was 75 ± 13 kg for AGA patients and 68 ± 11 kg for controls. The range of heights for all enrolled individuals was between 160 cm and 170 cm in both groups. Patients with polycystic ovary syndrome, hormonal replacement therapy, vitiligo, thyroid disease and autoimmune disease were excluded from the study. Demographical and clinical data of the enrolled individuals are summarized in Table 1. Informed consent approved by the internal review board were obtained from all enrolled individuals, and the study was conducted according to the Declaration of Helsinki Principles.

2.2 Genotyping APCDD1 Gene Polymorphisms

After the informed consent was obtained, peripheral blood samples were taken from the enrolled individuals, and genomic DNA was isolated by a BioRobot EZ1 DSP Workstation (QIAGEN; Godollo, Hungary). Coding regions and the flanking introns of the *APCDD1* gene were amplified with the PCR (polymerase chain reaction). Sequences used for primers were obtained from the UCSC Genome Browser (www.genome.ucsc.edu). Direct sequencing of the amplified PCR products was performed using an ABI Prism 7000 Sequencer (Life Technologies; Budapest, Hungary). The genotypes of the polymorphisms of the *APCDD1* gene were determined.

Table 1 Clinical data of enrolled individuals.

Clinical characteristics of enrolled individuals (n = 308)	Patients with AGA (n = 210)	Controls (n = 98)
Female to male ratio	1.7:1	2.1:1
Age (years)	45 ± 27	43 ± 25
Height (cm)	167 ± 9	163 ± 7
Weight (kg)	75 ± 13	68 ± 11
Body mass index (kg/m^2)	26 ± 4	25 ± 5

2.3 Genomic DNA, Total RNA and Protein Preparation from Tonsil Samples

As the APCDD1 protein is expressed at high levels in human tonsils and tonsils can be easily processed for protein studies, functional studies were carried out on tonsil samples. Informed consent was obtained from elective tonsillectomy patients affected by chronic tonsillitis, and tonsil samples (n = 20; 10 males and 10 females). Genomic DNA was isolated from the tonsil samples using the DNeasy Blood & Tissue Kit (QIAGEN). The fifth exon of the *APCDD1* gene was PCR amplified and sequenced. Genotype of the rs3185480 polymorphism of the *APCDD1* gene was determined for all tonsil samples. After identifying three tonsil samples from each genotype (homozygous common allele, heterozygous, homozygous rare allele, nine samples total), selected samples were processed for total RNA and protein extraction.

Total RNA from the nine selected tonsil samples was extracted using the RNeasy Fibrous Tissue Mini Kit (QIAGEN). Fragments overlapping exons 4 and 5 and exon 5 and the 3′ untranslated region were amplified by PCR and real-time quantitative RT-PCR. Primer and probe sequences were obtained from the Universal Probe Library Set (www.roche-applied-science.com). PCR product size was determined by gel electrophoresis.

Total protein extracts were prepared from the nine tonsil samples using the following protein lysis buffer: 1.5% sodium dodecyl sulphate (SDS), 62.5 mM Tris-HCl (pH 6.8), 5 mM ethylenediaminetetraacetic acid, 5% 2-mercapto-ethanol, 1 μg/mL antipain, 1 μg/mL chymostatin and 1 μg/mL leupeptin (all chemicals were obtained from Sigma-Aldrich, St Louis, MO, USA). Lysates were precleared by centrifugation and supernatants were stored at –20 °C. Concentration of the protein samples was determined using the BCA Protein Assay Kit (Santa Cruz Biotechnology; Budapest, Hungary). Protein quantities in sample volumes used for western blot were checked by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie brilliant blue (Sigma-Aldrich) staining.

2.4 Western Blot Analysis

Equal amounts of protein extracts of the tonsil samples (n = 9) were run on SDS-PAGE and transferred to nitrocellulose membrane (BioRad Laboratories, Hercules, CA, USA). Membranes were blocked by incubation in Tris-buffered saline (150 mM NaCl and 25 mM Tris, pH 7.4) containing 0.05% Tween 20 (Sigma-Aldrich) and 3% non-fat dry milk (Fluka Chemie AG, Neu-Buchs, Switzerland) for 2 h at room temperature and subsequently incubated overnight at 4 °C with anti-APCDD1 antibody produced in mouse (SAB1408369, Sigma-Aldrich) at a 1:100 dilution. In addition, a mouse monoclonal antibody (A5441, Sigma-Aldrich) at a 1:500 dilution was used to detect β-actin. Alkaline phosphatase-conjugated goat anti-mouse IgG (Sigma-Aldrich) was used as secondary antibody at a 1:4000 dilution in the blocking buffer for 2 h at room temperature. The blots were developed with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (Sigma-Aldrich) as a substrate. The expression profile of the protein samples was determined by semi-quantitative analysis using densitometry analysis of the intensity of bands on the western blots.

2.5 Statistical Analysis

The statistical significance of differences in the distribution of the *APCDD1* gene polymorphism genotypes was assessed by Chi^2-probe. Risk assessment for the development of AGA for carriers the rs3185480 polymorphism in heterozygous or in homozygous form was performed by nominal regression analysis. All statistical analyses were performed using SPSS software (IBM; Budapest, Hungary).

3. Results and Discussion

3.1 The Frequency of the Rare Allele of the rs3185480 Polymorphism Significantly Higher in AGA Patients

Genotypes of polymorphisms located in the coding regions of the *APCDD1* gene were determined for all enrolled individuals (n = 308). Of the investigated polymorphisms, only five, rs3748413, rs3748414, rs3748415, rs3185480 and rs60421798, had a rare allele frequency greater than 0.1 (Fig. 1). Since we were unable to perform statistical analysis for polymorphisms with a rare allele frequency less than 0.1, they were excluded from further analysis.

Three polymorphisms (rs3748413, rs3748414, rs3748415) are located in exon 3 of the *APCDD1* gene. These polymorphisms showed no difference in the distribution of the allele frequencies or the genotypes between AGA patients (n = 210) and controls (n = 98). As these polymorphisms had highly similar allele frequencies, linkage disequilibrium analysis was performed and confirmed that these polymorphisms belong to the same haplotype (r^2 = 1; Fig. 2.).

Two polymorphisms (rs3185480, rs60421798) are located in exon 5 of the *APCDD1* gene. Comparing the allele frequencies for AGA patients and controls, we found a significant difference (Chi^2-probe P = 0.0109) in the rare allele frequency of the rs3185480 polymorphism (Fig. 1.). We also compared the distribution of genotypes for the rs3185480 polymorphism between AGA patients and controls and found a significant difference (Chi^2-probe P < 0.001; Table 2).

Nominal regression analysis demonstrated a 3.5- and a 2.8-fold increased risk for the development of AGA for homozygote (confidence interval, CI, 0.933 – 13.125, P = 0.063) and heterozygote (CI 1.086 – 7.217, P = 0.033) carriers of the rare allele. The rs3185480 polymorphism results in a synonymous codon change: both alleles encode leucine and, thus, do not result in a change in the amino acid sequence of the APCDD1 protein (Fig. 3).

We did not detect an association with AGA of the rs60421798 polymorphism, also occurring in exon 5, based on allele frequency and genotype distribution.

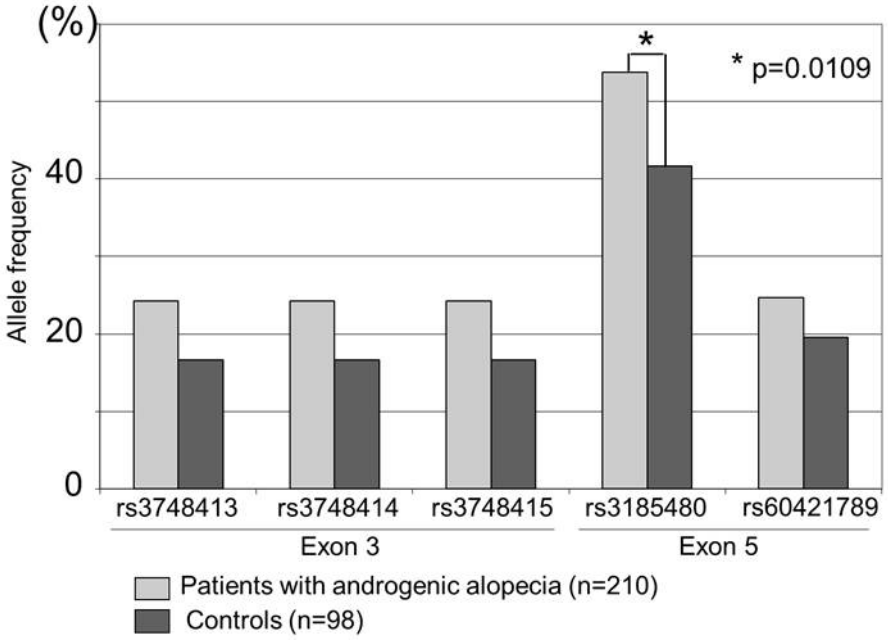

Fig. 1 Allele frequencies of polymorphisms located in the coding region of the *APCDD1* gene.
Five polymorphisms in *APCDD1* coding regions with rare allele frequencies greater than 0.1 were identified. Comparing the allele frequency for AGA patients with healthy controls, we found significant difference in the distribution of the rare allele of the rs3185480 polymorphism. The other investigated polymorphisms showed no association with AGA.

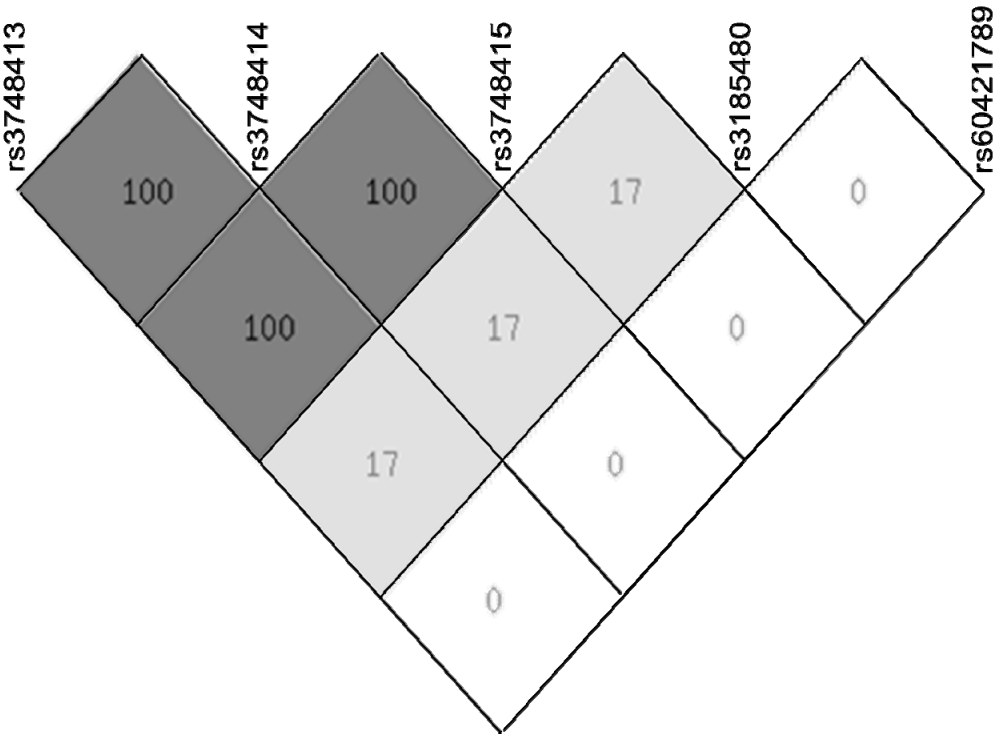

Fig. 2 Linkage disequilibrium analysis.
Three polymorphisms of exon 3 (rs3748413, rs3748414 and rs3748415) are inherited together. The two SNPs of exon 5 (rs3185480, rs60421798) are not involved in this haplotype.

Table 2 Distribution of rs3185480 genotypes.

Alleles at the rs3185480 polymorphism of the *APCDD1* gene	Homozygous common (CC) n (%)	Heterozygous (CT) n (%)	Homozygous rare (TT) n (%)
Patients with AGA (n = 210)	37 (18)	100 (48)	73 (35)
Controls (n = 98)	28 (29)	60 (61)	10 (10)

The two polymorphisms occurring in exon 5 did not exhibit linkage with each other or with the polymorphisms located in exon 3.

3.2 In Silico Analysis and the Assessment of Alternative Splice Variants

The rs3185480 polymorphism is a synonymous variant (c.1781C/T, p.Leu476Leu), and we desired to determine whether the sequence change has a functional consequence or whether it is simply a marker for AGA. Some synonymous changes have been reported to have a pathogenic role. In Netherton syndrome, for example, a synonymous mutation (c.891C/T, p.Cys297Cys) in the exon 11 of the SPINK5 gene, which alters an exonic splicing regulatory element, is causative for the disease [11]. Inspired by these data, *in silico* and *in vitro* mRNA analyses were carried out.

We performed an *in silico* prediction of exonic splicing regulatory elements for the region surrounding the rs3185480 polymorphism for both alleles using the ESEfinder 3.0 software (http://rulai.cshl.edu/cgi-bin/tools/ESE3/esefinder.cgi?process=home) and found that the rs3185480 polymorphism is located within a exonic splicing regulatory element: GCAGAGGACCTCGCAGAA (the position of the rs3185480 polymorphism is underlined). This result suggests that the rs3185480 polymorphism may alter the splicing of the *APCDD1* mRNA.

To confirm the *in silico* result, *in vitro* mRNA analysis was performed with tonsil samples that had been genotyped for the rs3185480 polymorphism. For

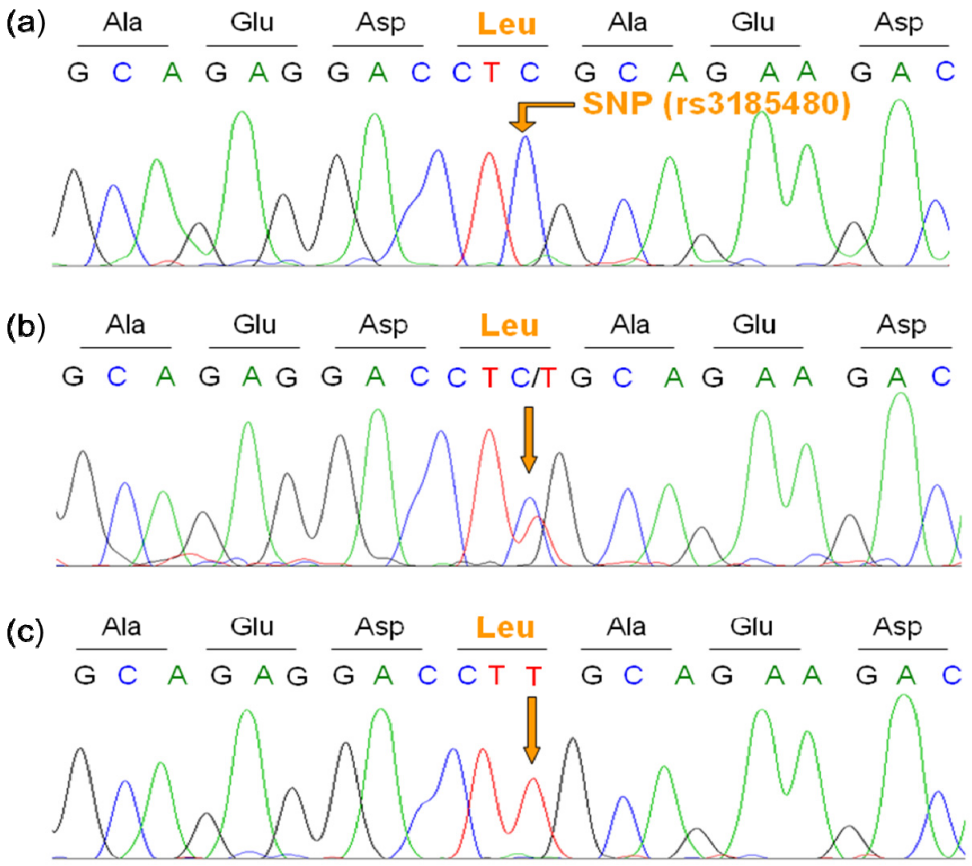

Fig. 3 The rs3185480 polymorphism of the *APCDD1* gene causes a synonymous codon change.
DNA sequencing results for the (a) homozygous common, (b) heterozygous and (c) homozygous rare alleles of the rs3185480 polymorphism.

each of the three genotypes of the rs3185480 polymorphism (homozygous common, heterozygous and homozygous rare), three independent samples were chosen (nine samples total). Fragment size was examined by RT-PCR and real-time quantitative RT-PCR for fragments amplified from a region that includes the splice junction between exons 4 and 5 and a region that includes part of exon 5 and the 3′ untranslated region of the *APCDD1* mRNA. No size difference was observed for the fragments amplified from tissues of all three genotypes (data not shown).

Although an *in silico* analysis of the *APCDD1* gene sequence suggested that the rs3185480 polymorphism might alter splicing, no difference in mRNA length was observed for rs3185480 genotypes. We conclude that it is unlikely that the rs3185480 polymorphism results in splicing variants.

3.3 Affects on Translational Efficiency by the Synonymous Codon Change for the rs3185480 Polymorphism

Since our mRNA studies could not answer the question how the rs3185480 polymorphism of the *APCDD1* gene contributes to AGA, further functional studies were performed. Most of the amino acids are coded by multiple codons: some of these codons occur with higher frequency in the human genome, while others with lower ones (http://www.genscript.com/cgi-bin/tools/codon_freq_table). For a given amino acid, for example for leucine CTC and CTG are the most common codons, encoding 60% of the total leucine in the human genome, while CTT, CTA, TTA and TTG altogether encodes only 40% of the total leucine. Codon-usage bias has been observed in almost all genomes and is

thought to result from selection for efficient and accurate translation of highly expressed genes [12, 13]. Codon usage is also implicated in the control of transcription, splicing and RNA structure [13]. Altered codon usage in the Neurospora FREQUENCY (FRQ) protein, for example, affects circadian clock function by influencing expression level, conformation, phosphorylation profile and stability of the FRQ protein [13].

Therefore, we investigated whether the altered codon usage of leucine due to the presence of the common or the rare allele of the rs3185480 polymorphism influences the translation efficacy and thus the level of the APCDD1 protein. To investigate this hypothesis, tonsil samples expressing the APCDD1 protein at high levels were used for Western blot analysis. The samples used in our protein studies were genotyped for the rs3185480 polymorphism and nine samples were chosen to represent each of the three genotypes with three independent samples.

APCDD1 protein levels were detected by western blot analysis for the genotyped tonsil samples and normalized to β-actin levels (Figs. 4a and 4b). Different levels of APCDD1 expression were observed for the three genotypes (Fig. 4c). Although the differences were not statistically significant, the tendency is clear and suggests the presence of the rs3185480 polymorphism is associated with decreased APCDD1 expression in a dose-dependent manner. Further immune-histochemical investigations of hair

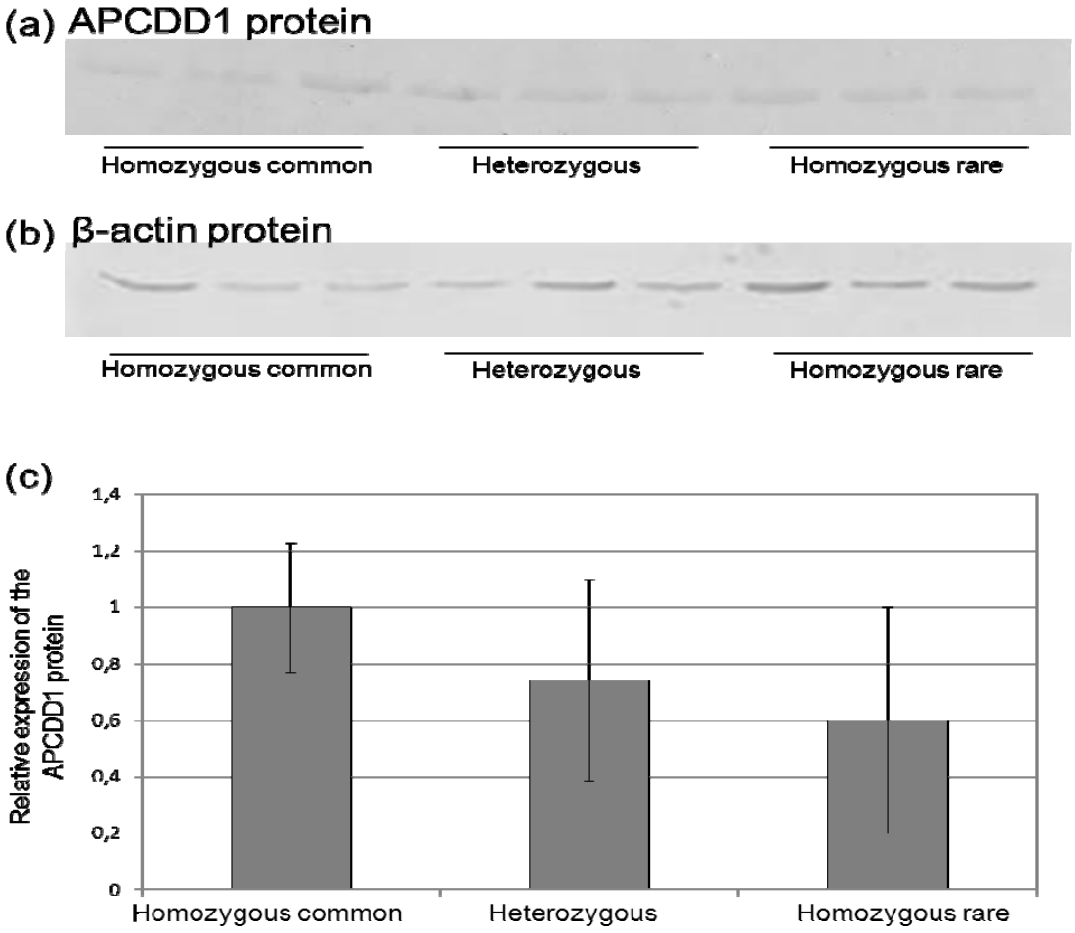

Fig. 4 Western blot analysis.

Expression profiles of (a) the APCDD1 and (b) the β-actin proteins in tonsil samples genotyped for the rs3185480 polymorphism. (c) Semi-quantitative analysis of western blots demonstrated decreased protein expression in tonsil samples for heterozygous and homozygous rare genotypes compared to samples with homozygous common genotype of the rs3185480 polymorphism.

follicles from individuals of all three rs3185480 genotypes are necessary to determine whether this polymorphism has the same effect on protein translation observed for tonsils. Results from such experiments could likely correlate rs3185480 genotype and AGA phenotype.

4. Conclusions

Here we report on the association of the rs3185480 polymorphism of the *APCDD1* gene and AGA. Since the rs3185480 polymorphism is a synonymous one and does not cause amino acid change on the sequence of the APCDD1 protein, we investigated whether this polymorphism can influence APCDD1 expression or it is a marker of AGA. Our *in silico* analysis suggested that the rs3185480 polymorphism might influence mRNA splicing since it is located in an exonic splicing regulatory element, however we could not prove this finding by *in vitro* mRNA studies. To further investigate the possible effect of the rs3185480 polymorphism on the expression level of the APCDD1 protein, we have performed Western blot analysis on tonsils samples previously genotyped for the investigated polymorphism and detected altered APCDD1 expression levels in samples carrying the rare alleles of the rs3185480 polymorphism. Functional data suggested that the rs3185480 polymorphism might alter translation efficacy and thus the level of the APCDD1 protein through altering codon usage of leucine from the preferred to a rare one. Further immune-histochemical investigations on the hair follicles of individuals with homozygous wild type, heterozygous or homozygous rare genotype of the rs3185480 polymorphism may answer whether this polymorphism has the same effect on protein translation we saw in the tonsils. This set of experiments would provide a direct link between the genotype of the rs3185480 polymorphism and the phenotype of AGA.

In summary, our results demonstrated that the synonymous rs3185480 polymorphism of the *APCDD1* gene is associated with the development of the most common hair disorder, AGA. Our functional analysis suggests that this polymorphism may influence the level of the APCDD1 protein through codon-usage bias.

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