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Determination of allelic expression of SNP rs1880676 in choline acetyltransferase gene in HeLa cells

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Abstract

Recently, we reported that several polymorphisms and haplotypes in the choline acetyltransferase gene (*ChAT*) are associated with nicotine dependence (ND). Of them, SNP rs1880676 is of particular interest because: 1) it is a non-synonymous variant located in the coding region of an alternatively spliced form of *ChAT* and 2) it is located in several haplotypes that are significantly associated with ND. The objective of this study was to determine, using an *in vitro* system, whether the alleles G (coding for aspartic acid) or A (coding for asparagine) of rs1880676 have any allele-specific effect on *ChAT* expression. We first used site-directed mutagenesis to construct two expression vectors differed in the allelic position of rs1880676(G/A), which were transfected into HeLa cells. We then measured expression of *ChAT* associated with each allele. We found significant expression differences for the two alleles, with the G allele being expressed significantly greater than A allele ($P<0.01$ at both mRNA and protein levels). Further, we validated the ChAT expression of the G allele was significantly higher than that of the A allele by using ELISA assay ($P=0.00016$). We concluded that rs1880676 is functional and that the allelic variations of this polymorphism are involved in developing ND by altering *ChAT* expression.

Keywords

choline acetyltransferase; rs1880676; expression; HeLa cells

INTRODUCTION

Cigarette smoking remains an important public health concern, as it is estimated to cause 435,000 deaths per year in the United States, which is 18.1% of all deaths [16]. Many of these deaths are caused by cancer, especially of the lung, and smoking is responsible for at

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least 30% of all cancer deaths. Also, because it is associated with numerous diseases of the cardiac, respiratory, and cerebrovascular systems [20], smoking imposes a significant financial burden on the health care system, accounting for more than $193 billion spent per year [4]. These economic and social costs continue to prevail despite decades of efforts to curtail tobacco use.

Of all tobacco users, more than 98% are cigarette smokers, and most are nicotine dependent [3]. Indeed, desire for nicotine, the primary addictive chemical in tobacco, is the main reason most smokers continue to smoke. Although most smokers want to quit, only 3%–5% are able to achieve sustained abstinence [3]. Prolonged use of tobacco products causes smokers to become increasingly physically dependent as a result of neurological changes caused by nicotine [2]. This dependence on nicotine has been identified as a phenotype heavily influenced by both genetic and environmental factors along with their interactions. The contribution of genetics to nicotine dependence (ND) has been established through many independent twin and family studies [14, 23], in which findings have shown at least a 50% heritability of ND in both male and female smokers [14].

Choline acetyltransferase (ChAT) represents a key component of the cholinergic system, where it is responsible for catalyzing the synthesis of an important neurotransmitter, acetylcholine [6, 19]. By modulating acetylcholine level, ChAT influences a wide range of brain functions such as learning, memory, and sleep, as well as control of muscular contraction [8]. Furthermore, modulatory effects of ChAT on mesocorticolimbic dopaminergic pathways involved in cognitive and affective function and a regulatory influence of ChAT on serotonergic and glutamatergic activities have been reported [1, 7]. Given the compelling evidence supporting a contribution of ChAT to various brain functions, studies have been conducted to examine whether variants in this gene are associated with schizophrenia [15] and Alzheimer's disease [9]. Although *ChAT* has been implicated in smoking [10], the involvement of this gene in ND was not demonstrated until our two recently reported genetic association studies, where we found that several variants and haplotypes in *ChAT* are significantly associated with both ND and smoking cessation [21, 24]. Of the SNPs analyzed in our previous studies, rs1880676, G/A, is attractive for further molecular examination because it is located in several haplotypes that are significantly associated with multiple measures of ND [21, 24]. Moreover, rs1880676 is a non-synonymous polymorphism located in the coding region of an alternatively spliced form of *ChAT* (S-transcript; Figure 1), which encodes a rarely studied isoform [18]. Considering the abovementioned facts about rs1880676 in the *ChAT* gene, it would be interesting to know whether the two alleles, G and A, of this polymorphism have allelic-specific effects on the expression of the S-transcript of *ChAT*, which was the main objective of this study.

MATERIALS AND METHODS

Plasmid construction and mutagenesis

The cytomegalovirus-based expression vector pRBG4 [13] containing the S transcript of *ChAT* (pRBG4-S) was kindly provided by Dr. Andrew Engel of the Mayo Clinic [18]. The entire DNA of the pRBG4-S plasmid was sequenced at the University of Virginia DNA Sequencing facility, which confirmed the presence of the A allele of rs1880676 (A/G). The G allele was generated using the Quikchange II Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA) with the following two primers (Operon, Huntsville, AL): 5′-gtggccggaatgcagaaatgaagcactgagcac-3′ (forward) and 5′-gtgctcagtgcttcatttctgcattccggccac-3′ (reverse). Primers were designed with the Quikchange Primer Design Program (www.stratagene.com/qcprimerdesgin). The mutated construct containing the G allele of rs1880676 was confirmed by sequencing.

Cell culture and transfection

The immortal HeLa cell line was purchased from the American Type Culture Collection (Manassas, VA) and cultured in Dulbecco Modified Eagle Medium with high glucose (Hyclone, Logan, UT), 10% fetal bovine serum, and 1% antibiotics (Mediatech, Herndon, VA) at 37°C in 5% CO_2. The transfections of A and G allele constructs and the empty pRBG4 vector (negative control) were carried out with Lipofectamine 2000 from Invitrogen (Carlsbad, CA, USA), according to the manufacturer's protocol. After 48 hours of transfection, cells were harvested for purification of RNA, protein, or cell extracts. For each plasmid constructed, quadruplicate transfections were performed simultaneously, and their extents of expression were assayed separately and then averaged in each experiment. Three independent experiments were conducted for replication purposes. Except that we examined a different polymorphism in this report, all experiments reported here were conducted under essentially the same conditions reported previously by our group [5, 11, 26].

mRNA expression analysis

Total RNA was extracted from transfected HeLa cells with Trizol reagent (Invitrogen). Potential plasmid DNA was treated with RNase-free DNase I (Ambion, Austin, TX) at 37°C for 30 min prior to reverse transcription. The RNA was reverse-transcribed with SuperScript IIRT (Invitrogen) to obtain cDNA. The transcribed cDNA was then assayed with *TaqMan* Gene Expression Assays (Applied Biosystems, Foster City, CA) designed specifically for measuring *ChAT mRNA* expression. The custom *TaqMan ChAT* probe was created with the following two primers: 5′-ccagagatgtggccggaatg-3′ (forward) and 5′-cctggtgcagggatgca-3′ (reverse) using the Custom *TaqMan* Assay Design Tool (https://www5.appliedbiosystems.com/tools/cadt/) and applied with the standard *TaqMan* PCR procedures in the ABI Prism 7900 HT Sequence Detection System. Although the S-transcript encodes both the 70-kDa and 74-kDa isoforms, the *TaqMan* probe used in the current study was designed to bind to the exon specific to 74-kDa ChAT, which contains rs1880676 of interest; this will ensure that the probe was not binding to a region of cDNA shared by the two isoforms. GAPDH was used as an internal control to normalize the expression of *ChAT*.

Western blotting analysis

Total protein was extracted from HeLa cells by homogenization with a syringe and needle in RIPA buffer (Santa Cruz, CA), and the protein concentration was determined by the Bio-Rad assay (Bio-Rad Laboratories, Hercules, CA). For each sample, 10 μg of total protein was subjected to 10% SDS-PAGE and then electrophoretically transferred to nitrocellulose membranes overnight at 25 V. The membrane was incubated with 1% BSA in TBST buffer at room temperature for 1 h and then for 1.5 h in blocking buffer containing goat anti-choline acetyltransferase antibody (dilution 1:1,000; Millipore Corporation, Temecula, CA). After three washes for 10 min each in TBST buffer, the membrane was incubated with secondary antibody, anti-goat IgG (1:5,000; Sigma-Aldirch), for 1.5 h at room temperature. The hybridized membrane was washed three times with TBST buffer for 10 min each and then exposed to X-ray film. Tubulin was used to normalize the protein concentration and test the loading efficiency of each sample. In Western blotting analysis, mouse monoclonal antibody to α-tubulin (1:5000) was used as the primary antibody and anti-mouse IgG (1:5000) as the secondary antibody.

Enzyme-linked immunoabsorbent assay (ELISA) analysis

To further quantify ChAT level associated with each allele in the transfected HeLa cell lystates with plasmids carrying different allele of rs1880676, we also performed ELISA analysis with the kit purchased from the Mybiosource Inc. (San Diego, CA) based on the

protocol provided by the manufacturer. The detection range, as specified in the manufacturer's manual, was from 15.6 pg/ml to 1000 pg/ml. The samples were measured with a microplate reader at a wavelength of 450 nm and a wavelength correction of 540 nm.

Statistical analysis

By using a custom-designed TaqMan *ChAT* probe, we performed quantitative RT-PCR analysis of plasmids containing either the A or the G allele and the empty pRBG4 vector as a negative control in three independent experiments with four replicates for each experiment. To compare the RNA expression of the two alleles of rs1880676, the comparative C_t method was utilized with the GAPDH as control [25]. First we normalized the C_t values obtained for each sample expressing A, G or pRBG4 vector, with the C_t values for internal control GAPDH. The normalized C_t values for A and G alleles were then adjusted with that of pRBG4 vector.

For the protein level and concentration in transfected HeLa cells, the mean fold change values were calculated for all A and G allele-specific samples and normalized to appropriate controls as specified in each experiment. Student's two-tailed *t*-test was used to analyze the data in order to determine if a significant difference existed between the fold change values of the two alleles of rs1880676. GraphPad Prism 4 software (GraphPad, San Diego, CA) was employed to perform all statistical analyses. $P<0.05$ was considered statistically significant.

RESULTS

Comparison of *ChAT mRNA* expression of A and G alleles of rs1880676 in HeLa cells

Because HeLa cells do not express endogenous ChAT [17], we chose it for determining the expression of these two alleles in this study. Statistical analysis revealed that the mean fold change for the A allele samples was 0.971 ± 0.095 and that for the G allele samples was 2.044 ± 0.312, indicating that expression of the G allele of rs1880676 is about 2.1-fold higher than that of the A allele ($p<0.0001$; Figure 2).

Comparison of ChAT level of A and G allele of rs1880676 in HeLa cells

To determine whether the difference in the allele-specific mRNA was translated into protein, we performed Western blotting analysis for three independent replications with four replications for each experiment to measure the ChAT protein expression of the G and A alleles. After normalization to the corresponding tubulin expression of each sample, we found that the G allele (3.160 ± 0.095) showed significantly higher expression than the A allele (2.390 ± 0.252) ($p = 0.0077$; Figure 3). These results again indicate that the expression of the G allele is significantly higher (ca. 1.32-fold) than that of the A allele at the protein level.

Comparison of ChAT concentrations of A and G allele of rs1880676 in HeLa cells

To more accurately determine the ChAT expression difference associated with each allele of rs1880676, we performed ELISA for three independent experiments with four replicates for each plasmid construction to measure the ChAT concentration of the G and A alleles. Our results showed that the concentration of G allele (568.12 ± 92.27; pg/ml) was significantly higher (ca. 2.67-fold) than that of the A allele (212.54 ± 76.71; pg/ml), with a *p* value of 0.00016 (Figure 4).

DISCUSSION

In this study, we showed differential allelic effects of rs1880676 (G/A) on the expression of *ChAT* at different levels in HeLa cells, which represents a logical extension of our previous studies, where we showed several variants and haplotypes in *ChAT* gene are significantly associated with both smoking cessation and ND [21, 24]. Among the SNPs examined in our previous association studies, rs1880676 appeared to be an attractive candidate for further functional study based on our genetic association results and its physical location within *ChAT*.

In addition to the genetic association results, where we found several haplotypes where SNP rs1880676 locations are significantly associated with ND, the physiological location of the SNP in the coding region of the S-transcript of *ChAT* was another motivation for us to investigate this polymorphism. The S-transcript of *ChAT* encodes two isoforms, 74-kDa and 70-kDa ChAT [18]. Although 70-kDa ChAT is the common form that catalyzes the synthesis of acetylcholine and locates to the cytosol of cells [22], there is an alternatively spliced 74-kDa ChAT form that has rarely been studied. Whereas rs1880676 is found in an intron of 70-kDa ChAT, the SNP is located in the exon of 74-kDa *ChAT* because of an additional 36 N-terminal residues, such that rs1880676 acts as a non-synonymous SNP that changes amino acid 7 from aspartic acid to asparagine (see Figure 1). Despite its potentially functional role, the characterization of rs1880676 by expression level has yet to be uncovered [21, 24]. We hypothesized that rs1880676 is a functional polymorphism that affects the expression of *ChAT*.

Our findings provide evidence that a significant allelic difference exists at various *ChAT* levels for rs1880676, with the G allele being expressed more highly than the A allele. This strongly indicates that altered ChAT activity and expression levels contribute to some extent to the development of ND. We also performed bioinformatics analysis using the SIFT program (http://sift.jcvi.org) [12] to predict whether amino acid substitutions affect protein function. This analysis revealed that rs1880676 would be damaged at the protein level when the amino acid residue is changed from aspartic acid (G allele) to asparagine (A allele). In conjunction with the findings from the association study and bioinformatics analysis, we here provide convincing evidence that rs1880676 is a functional polymorphism that affects *ChAT* expression. Importantly, we showed such allelic expression differences of rs1880676 were detected at both RNA with quantitative RT-PCR and protein levels with Western blotting and ELISA analysis in transfected-HeLa cell extracts, confirming that this is a highly important variant with likely significant kinetic activity in ChAT and possibly ND. By comparing those detected fold difference between the two alleles with three molecular techniques (i.e., qRT-PCR for RNA, and Western and ELISA for protein), we found the fold difference detected by ELISA assay (2.67fold) is more similar to the fold difference detected by qRT-PCR (2.10-fold) than by Western blotting analysis (1.32-fold), confirming the value of conducting ELISA assay in this study and its expected accuracy compared with Western blotting analysis. In addition to its association with ND, rs1880676 and other variants in ChAT have been significantly associated with schizophrenia and the response to antipsychotic drug treatment [15], as well as with Alzheimer's disease [9].

In summary, we demonstrated that SNP rs1880676 is a functional variant, of which the G allele is expressed to a greater extent than the A allele at the mRNA and protein levels. From these findings and previous association analysis of the *ChAT* gene with ND, we conclude that this gene plays an important role in the etiology of ND. Since this conclusion was derived primarily from an *in vitro* system, thus it is important to determine whether such allelic specific expression difference can also be detected *in vivo*. Moreover, further studies

with independent human smoker samples are needed to confirm our association findings as well as to understand the molecular mechanisms of the action of this functional variant.

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Highlights

- Choline acetyltransferase (ChAT) influences a wide range of brain functions.
- Variants in *ChAT* are associated with nicotine dependence (ND).
- SNP rs1880676 is a non-synonymous variant located in the code region of *ChAT*.
- The G allele of rs1880676 is expressed significantly higher than A allele at RNA and protein levels.
- SNP rs1880676 is functional variant that plays a significant role in developing ND.

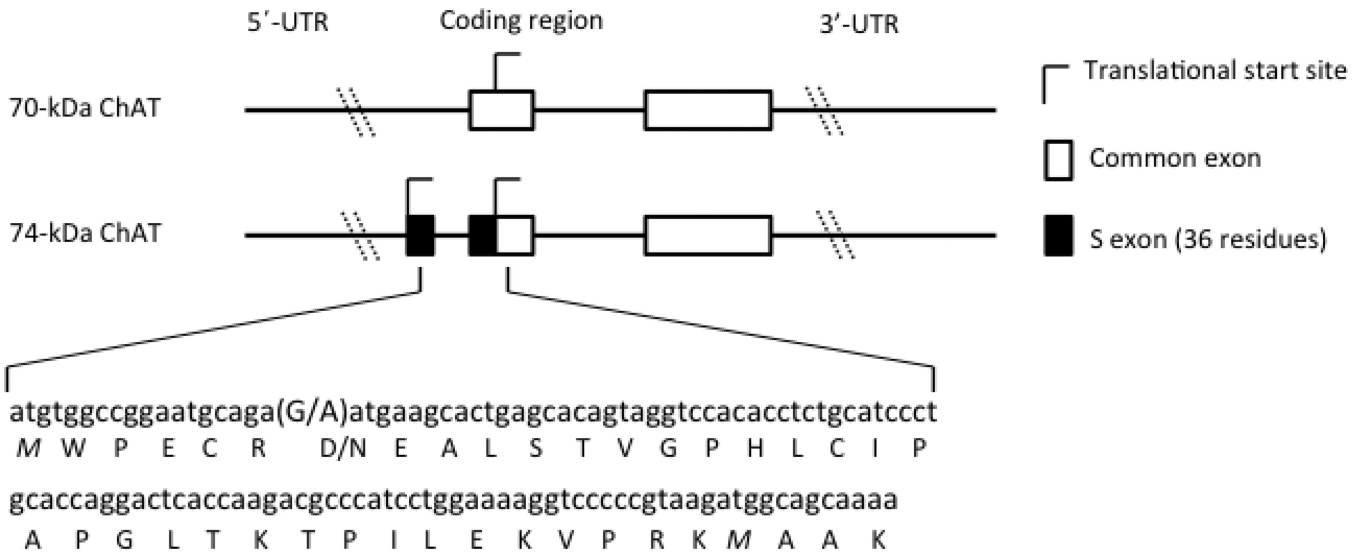

Figure 1.

Coding region differences between 70-kDa and 74-kDa ChAT. Although both isoforms are encoded by the S-transcript of ChAT, 74-kDa ChAT contains a 36-amino acid N-terminal extension, in which the minor allele of rs1880676 (G/A) alters the seventh amino acid from aspartic acid to asparagine (D → N). The rs1880676 SNP is located in an exon specific to 74-kDa ChAT.

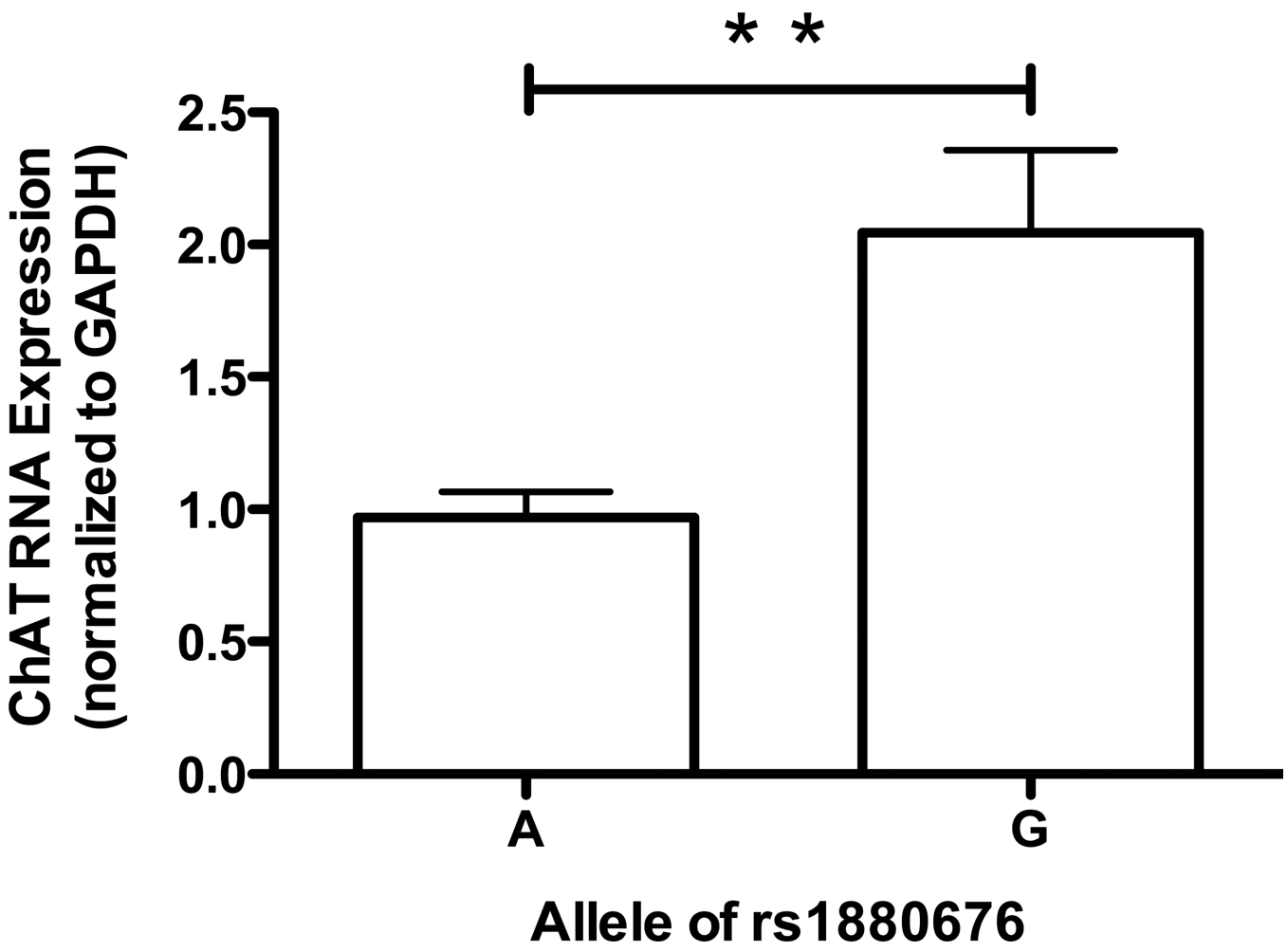

Figure 2.

mRNA expression of alleles A and G of rs1880676. Analysis byqRT-PCR revealed that the mean fold change of the G allele was significantly higher than that of the A allele. Representative data are shown as mean ± SD (*p <0.01, **p <0.001; n = 5–8/group).

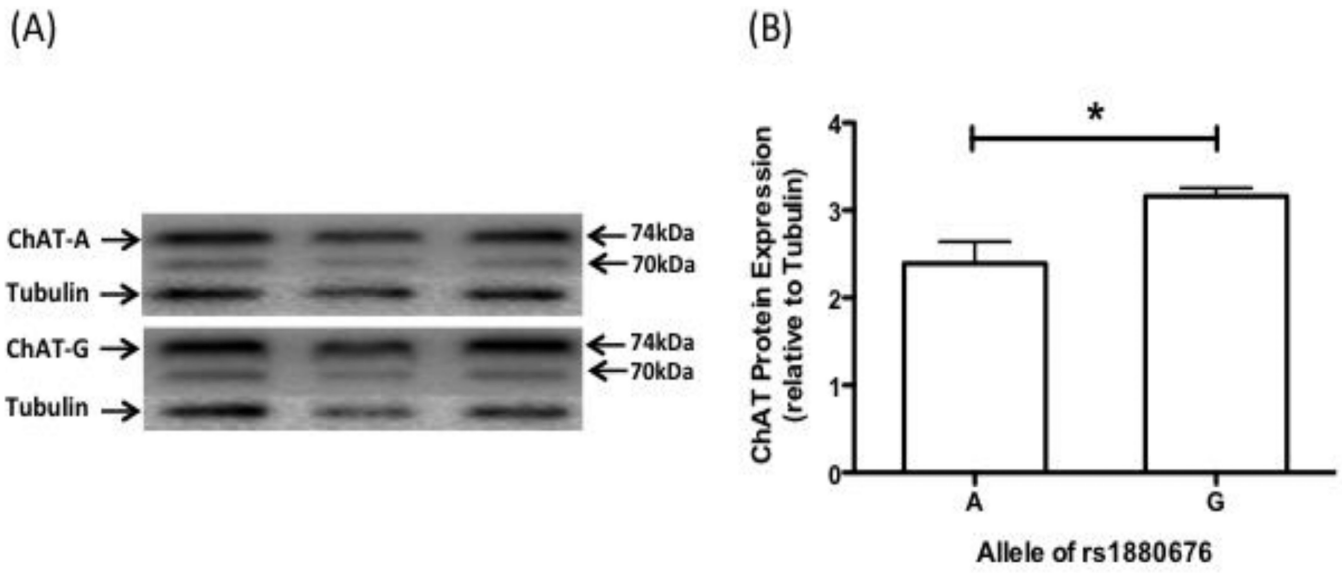

Figure 3.

Expression analysis of A and G allele of rs1880676 at the protein level. (A) Representative Western blotting images for alleles of rs1880676 and beta-tubulin (used to normalize expression of each allele among samples). (B) Western blotting analysis revealed that the G allele was expressed to a significantly greater extent than was the A allele. Data in panel (B) are given as means ± SD (*p <0.05; N = 4/group).

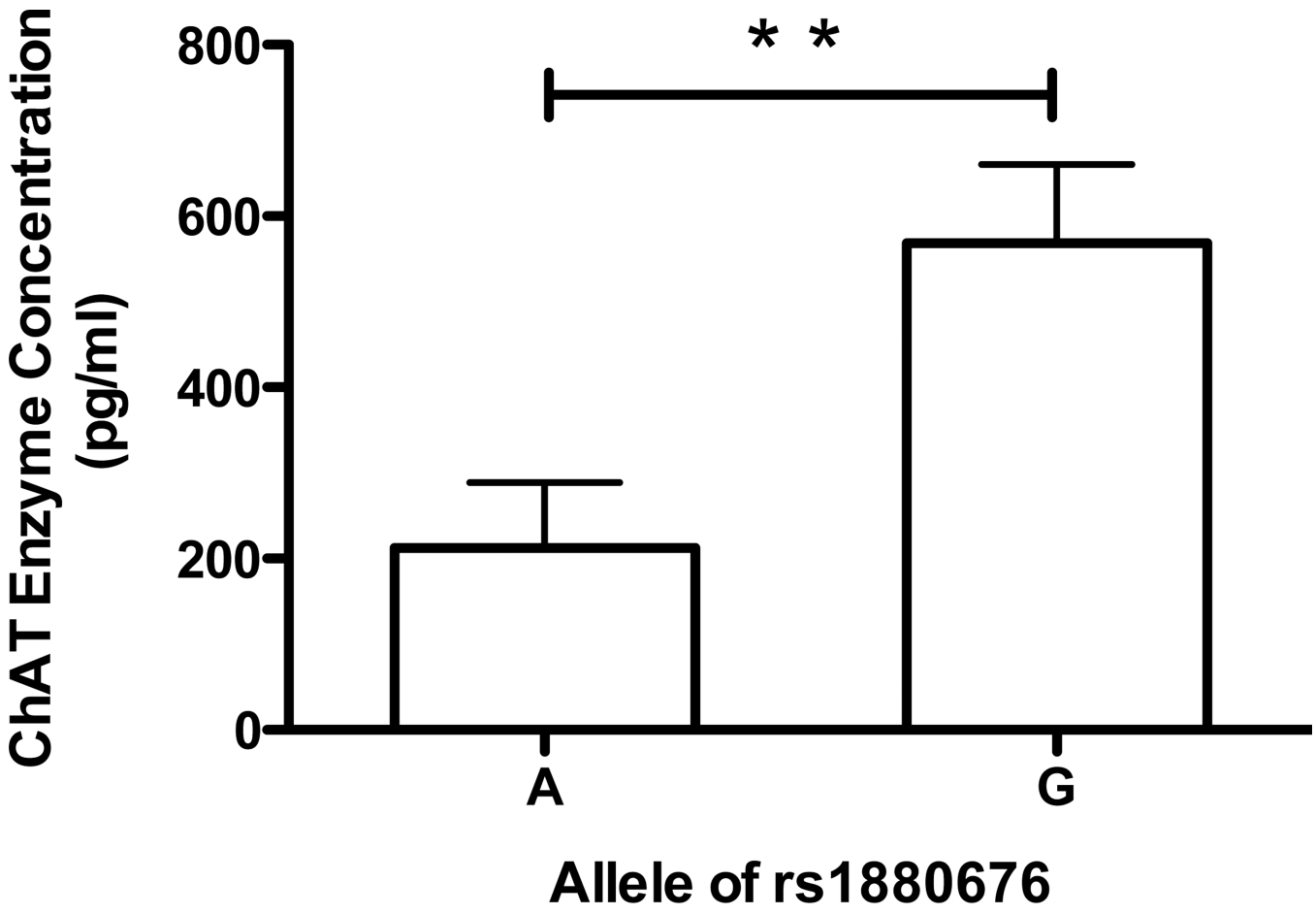

Figure 4.

Protein concentration analysis of A and G allele of rs1880676. Based on a standard curve generated from the known ChAT concentration provided in the ELISA kit, we calculated the protein concentration of each sample for both A and G allele followed by statistical analysis. Our ELISA assay revealed that the concentration of the G allele was significantly higher than that of the A allele. Data shown are means ± SD (**$p < 0.001$; N = 5/group).