Association between the *ABCC11* gene polymorphism and the expression of apolipoprotein D by the apocrine glands in axillary osmidrosis

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Abstract. It has been suggested that the adenosine triphosphate-binding cassette sub-family C member 11 (*ABCC11)* gene polymorphism and apolipoprotein D (ApoD), an odor precursor carrier, may be important in the formation of axillary odor. To date, few studies have examined the potential correlation between these two factors. The present study aimed to investigate the association between a 538 G>A single-nucleotide polymorphism (SNP) of the *ABCC11* gene and the mRNA expression levels of *ApoD* in the apocrine gland of patients with osmidrosis. The 538 G>A polymorphism genotypes of 33 patients with a clinical diagnosis of osmidrosis were analyzed by polymerase chain reaction (PCR) and a base-quenched probe method, and they were divided into two groups according to the results. The G allele functions as a dominant gene; therefore, patients with the GG or GA genotype were allocated to Group I (n=28) and patients with the AA genotype to Group II (n=5). The mRNA expression levels of *ApoD* in the apocrine glands were determined by reverse transcription-PCR. The results indicated that the mRNA expression levels of *ApoD* were significantly higher in the apocrine glands of patients in Group I compared with those in Group II (P<0.01). In conclusion, the results indicated that the *ABCC11* gene SNP of the 538 G>A allele was associated with a downregulation of the mRNA expression of *ApoD* in the apocrine glands, which may indicate a role for the *ABCC11* gene in the mediation of osmidrosis by enhancing the transition of odor precursors via the ApoD pathway.

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Introduction

Osmidrosis is one of the most common complaints in the departments of plastic surgery at the First Affiliated Hospital of Nanjing Medical University (Nanjing, China). The apocrine glands, which are located in the axilla, are the predominant cause of axillary odor (1). This is due to the fat-like secretion produced by the apocrine glands, which is broken down into volatile odorous substances by bacteria (2).

Certain factors affect and/or contribute to the formation of axillary odor, including genetics, gender, age, developmental stage, diet, treatment history and family history (3-5). However, the mechanisms underlying the development of osmidrosis remain to be elucidated. A previous study demonstrated that genetic and environmental factors are considered the most significant factors in the development of osmidrosis (6). The effect of genetics as a vital contributing factor is also of interest. Increasing evidence has indicated that the single nucleotide polymorphism (SNP), rs17822931 (538 G>A), of the *ABCC11* gene located on human chromosome 16q12.1 is associated with axillary osmidrosis (3). *ABCC11* is expressed and localized in the apocrine glands and has a key function in the secretion of odorants and their precursors (7). A previous study revealed that ~98.7% of individuals with osmidrosis have the GG or GA genotype (7), which is a significantly larger proportion compared with the overall population and suggests that the G allele of *ABCC11* may be important in the expression of axillary odor. Apolipoprotein D (ApoD), a 29 kDa glycoprotein, is the primary protein component of high-density lipoprotein in human plasma (8). It has been demonstrated that ApoD is a physiological carrier of odor precursors *in vivo* and its sequence is also expressed in the apocrine glands (9), indicating a role for ApoD in the transition of axillary odor precursors. Furthermore, it has been revealed that levels of ApoD are increased in individuals with osmidrosis (4). Associations between the rs17822931 SNP of *ABCC11,* ApoD and axillary odor have been observed.

Notably, the ethnic distribution of the *ABCC11* G allele is similar to that of high-level apocrine ApoD (10). To date, few studies have investigated the correlation between *ABCC11* and *ApoD*. Therefore, the present study aimed to further examine the association between the *ABCC11* genotype at rs17822931 and the mRNA expression levels of *ApoD* in the apocrine glands of patients with osmidrosis.

Materials and methods

Patients and samples. All procedures were performed according to protocols approved by the Ethical Review Board of the First Affiliated Hospital of Nanjing Medical University (Nanjing, China) between November 2012 and August 2013. A total of 33 patients exhibiting symptoms of axillary odor and receiving surgery in the Department of Plastic and Burn Surgery (First Affiliated Hospital of Nanjing Medical University) were included in the study following the provision of written informed consent. Peripheral blood (2 ml) was drawn from all subjects using into EDTA-containing tubes (BD vacutainer; BD Biosciences, Franklin Lakes, NJ, USA) and stored at -20˚C to obtain genomic DNA. The gender, age, height, weight, dietary preferences, treatment history and family history of each patient were recorded. To evaluate the odor severity, the patients were required to bathe 1 day prior to their procedure and rest in an examination room (22˚C) for 30 min prior to surgery. The entire axilla was then exposed for odor evaluation, which was performed by a researcher and plastic surgeon. Four distinct stages were outlined according to the distance from which the odor was sensed, which are outlined in Table I. When there was a disagreement in stage designation, the stage was evaluated by an additional researcher and the median was used. All the patients received surgery involving micro-incision subcutaneous trimming under local anesthesia (Lidocaine, 2% diluted with saline to 1%; Shanghai Fuxing Chaohui Pharmaceutical Co., Ltd., Shanghai, China). The subcutaneous tissues, including the apocrine glands of the axilla, were removed during surgery, immediately frozen in liquid nitrogen (Changzhou Changyu Practical Gas Co., Ltd., Changzhou, China) and transferred for storage at -80˚C.

Table I. Stages of odor severity.

Stage	Distance from odor detection (cm)
One	<15
Two	≥15 and <30
Three	≥30 and <100
Four	≥100

Base-quenched probe genotyping of the ABCC11 (538 G>A) polymorphism. Individual genomic DNA was extracted from 250 μl samples of peripheral blood using a 3S Blood DNA Isolation kit (Shenergy Biocolor Co., Shanghai, China), according to the manufacturer's instructions. Sequence data for the human *ABCC11* gene was obtained from the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov/gene/85320#reference-sequences). Primer Premier 5.0 (Premier Biosoft International, Palo Alto, CA, USA) was used to design a pair of primers and a probe to distinguish the nucleotide (538 G>A). The primer and probe sequences are shown in Table II. All the specific primers and probe were synthesized and fluorescence-modified by Sangon Biotech, Co., Ltd. (Shanghai, China). *Taq* DNA polymerase, 4X deoxyribonucleotide triphosphates (dNTPs), 10X polymerase chain reaction (PCR) buffer (100 mM Tris-HCl, pH 8.3 at 25°C; 500 mM KCl; 15 mM MgCl2; and 0.01% gelatin) and $MgCl_2$ were purchased from Shenergy Biocolor Co. Briefly, PCR was performed as using 2 μl genomic DNA template, 2.5 μl 10X PCR buffer, 1.5 μl 25 mM $MgCl_2$, 0.5 μl 4X dNTPs, 1.25 U *Taq* DNA polymerase, 0.1 μl 100 μM of each primer and 0.2 μl 10 μM probes in a final reaction volume of 25 μl. Thermal cycling for *ABCC11* (538G>A) was performed on a LightCycler (Version 480II; Roche Diagnostics, Basel, Switzerland) under the following conditions: 5 min of initial denaturation at 95˚C, followed by 40 cycles at 95˚C for 1 sec (temperature transition rate 4.4˚C/sec), 62˚C for 25 sec and 72˚C for 10 sec. The analytical melting program involved melting the PCR products at 95˚C for 1 min, 35˚C for 2 min and increasing the temperature to 70˚C at a transition rate of 0.06˚C/sec, with continuous acquisition of fluorescence data using the LightCycler, as previously described (11). The sequences of the homozygous G genotype and the base-quenched probe formed an exact match resulting in a higher melting temperature (TM) to enable the different genotypes to be distinguished (12). Subsequently, seven samples were randomly selected and sequenced on an automatic sequencer (Model 3730, Applied Biosystems, Invitrogen Life Technologies, Shanghai, China) to verify the genotyping results.

Reverse transcription-PCR analyses of the mRNA expression of ApoD. The total RNA of apocrine gland tissues were extracted using the Total RNAPurification kit (Sangon Biotech Co., Ltd, Shanghai, China), according to the manufacturer's instructions, no prior steps were required. The quality of the RNA samples was evaluated by measuring the absorbance at 260/280 nm (Biophotometer; Eppendorf, Hamburg, Germany). Total RNA (2 μg) was reverse transcribed to cDNA using a RevertAid First-Strand cDNA Synthetic kit (Thermo Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Sequence data for human *ApoD* mRNA was obtained from the NCBI database. Primer Premier 5.0 software was used to design the primers and probes for the human *ApoD* and *GAPDH* genes. The primers and probes are shown in Table III. *GAPDH* was used as the reference gene. Amplifications were performed in a 7300 Real-time PCR system (Applied Biosystems Life Technologies, Foster City, CA, USA). The optimum reaction conditions were obtained using 2.5 μl 10X PCR buffer, 2.5 μl 25 mm $MgCl_2$, 0.5 μl 10 mm 4X dNTPs, 0.25 μl 5 U/μl *Taq* DNA polymerase, 0.1 μl 100 μm sense primer, 0.1 μl 100 μM antisense primer, 0.1 μl 100 μM probe and 2 μl template cDNA. Finally, 16.95 μl ddH_2O was added to the reaction mixture. Standard cycling conditions were as follows: 95˚C for 3 min, followed by 40 cycles at 95˚C for 5 sec (temperature transition rate 4.4˚C/sec), 60˚C for 27 sec and 40˚C for 1 min. Changes in transcriptional expression were estimated using the $2^{-\Delta\Delta CT}$ method (13). Significant differences in gene transcription were evaluated using Student's t-test.

Table II. Sequences of primers and probe of ABCC11 gene (538 G>A).

Primer/probe	Primer sequence (5'-3')
Forward primer	gattccaccagttccattatcctct
Reverse primer	cccccaaacctcaccaagtct
Probe	agtgtactcgggccagta-FAM

FAM represents 6-carboxyfluorescein and the underline indicates the polymorphic nucleotide (538 G>A).

Table III. Primers and fluorescent probes for quantitative polymerase chain reaction.

Gene/primer/probe	Sequence (5'-3')
ApoD	
Forward	ccagtcaccaagacaggcatc
Reverse	ctggagaagggacctggagc
Probe	FAM-atcggctgattctgcatctggaaact-TAMRA
GAPDH	
Forward	ggaaggtgaaggtcggagtc
Reverse	cgttctcagccttgacggt
Probe	FAM-tttggtcgtattgggcgcctg-TAMRA

FAM and TAMRA represent 6-carboxyfluorescein and the quencher, respectively.

Statistical analysis. Statistical analyses were performed using SPSS version 13.0 (SPSS, Inc., Chicago, IL, USA). $P<0.05$ was considered to indicate a statistically significant difference. Quantitative data was evaluated to determine whether the various data sets were normally distributed. Age and BMI of patients were expressed as mean ± standard deviation and ApoD mRNA levels were expressed as median with interquartile range. Student's t-test was used for two-group comparisons of normally distributed data sets, and abnormally distributed data sets were modified by square root transformation prior to comparison. Fisher's exact test was applied to graded data.

Results

Genotyping of ABCC11 538 G>A polymorphisms using the base-quenched probe method. The biallelic SNP marker (G/A) of *ABCC11* 538 G>A yielded three distinct genotypes, including G homozygote, A homozygote or GA heterozygote. The homozygous G genotype and the base-quenched probe had corresponding base pairs, resulting in a high melting temperature (TM). When the homozygous A genotype was present, a mismatch in the base pairs occurred and the TM was reduced, as previously described (11). The heterozygous genotype was characterized by two TM valleys (Fig. 1). In the 33 patients, the GG, GA and AA genotypes were present in 1,27 and 5 individuals, respectively. As the allele G was the dominant gene, the patients were divided into two groups:

Table IV. Patient characteristics.

Characteristic	Value
Number of patients (n)	33
Gender, n (%)	
Male	9 (27.27)
Female	24 (72.73)
By group (male/female; n)	I (9/19)
	II (0/5)
Age (years)	
Mean	24.44±6.76
Range	16-44
By group	I (24.22±7.01)
	II (25.60±5.73)
BMI (kg/m^2)	
Mean BMI	21.35±3.00
By group	I (21.48±3.10)
	II (20.70±2.57)
Family history, n (%)	
With osmidrosis	24 (72.73)
Without osmidrosis	9 (27.27)
By group (with/without; n)	I (23/5), II (1/4)
Dietary preference, n (%)	
Meat or capsicum	17 (51.52)
No meat or capsicum	16 (48.48)
By group (meat or capsicum/none; n)	I (14/14)
	II (3/2)
Odor severity stage, n (%)	
One	5 (15.15)
Two	8 (24.24)
Three	11 (33.33)
Four	9 (27.27)
By group (one/two/three/four; n)	I (2/7/10/9)
	II (3/1/1/0)

Values are expressed as the mean ± standard deviation, unless otherwise stated. BMI, body mass index; I, GG/GA genotype; II, AA genotype.

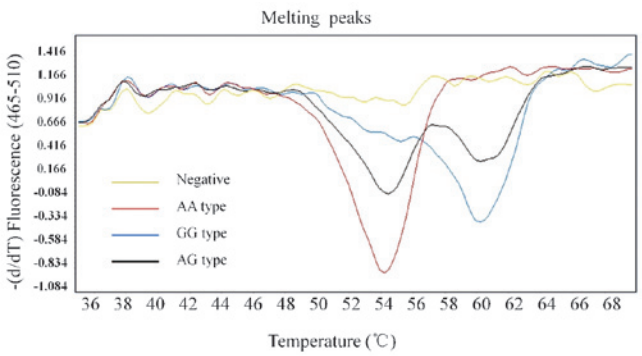

Figure 1. Derivative melting curves (-dF/dT vs. temperature) for the identification of the three genotypes. The derivative melting valleys are oriented in negative scale to facilitate visualization of TMs. The TM of the GG genotype was ~60˚C, AA genotype was ~54˚C and the GA heterozygote exhibited two melting valleys at 60 and 54˚C. TM, melting temperature; -dF/dT, negative derivative of fluorescence/temperature.

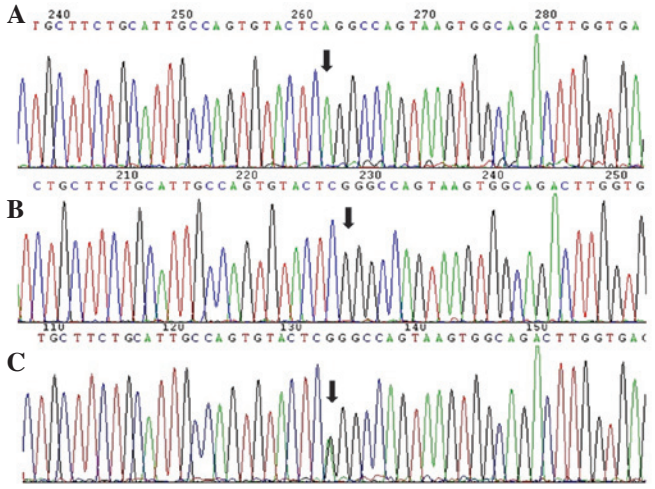

Figure 2. Gene sequencing using an automatic sequencer. (A) Sequences identified as AA genotype. (B) Sequences identified as GG genotype. (C) Sequences identified as AG genotype. The arrows indicate the mutation site.

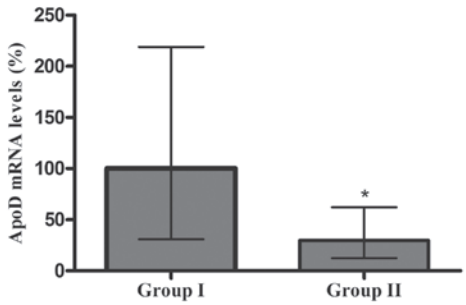

Figure 3. mRNA levels of *ApoD* determined by reverse transcription polymerase chain reaction in Groups I and II. Data are expressed as the median and interquartile range. Group I levels are presented as 100%. *P<0.05, vs. Group I.

Group I, comprising individuals with the GG or GA genotype and Group II, comprising individuals with the AA genotype. Complete concordance was observed between the results of the DNA sequencing and the base-quenched probe method (Fig 2).

Differences in other factors between the groups. As indicated in Table IV, statistically significant differences (P<0.05) were observed in gender, family history and odor severity between the groups, whereas no significant differences (P>0.05) were observed in age, body mass index (BMI) or diet between the groups.

PCR analysis of the mRNA expression of ApoD. As shown in Fig. 3, the relative mRNA expression levels of ApoD were significantly higher in the Group I (100.0%, interquartile ranged from 35.96% to 217.5%) compared with those in the Group II (28.38%, interquartile ranged from 11.98% to 59.11%; P<0.05).

Discussion

Osmidrosis is a hereditary condition (14). It has been demonstrated that the 538 G>A (Gly180Arg) SNP of the *ABCC11* gene is associated with axillary odor in the Japanese population (7), in which individuals with the AA genotype exhibited elimination of odor. This mutation has previously been recommended as a diagnostic standard (7), however, odor is a subjective factor and is readily affected by several other factors, including hormones (4), the environment (6) and dietary intake (5). As a physiological factor, the effects of genetic mutations do not take these factors into account and, therefore, the genetic diagnosis of osmidrosis may be inaccurate.

Axillary odor is comprised of a mixture of numerous substances, among which (E)-3-methyl-2-hexenoic acid (E-3M2H) is one of the key odorous components (15). ApoD is involved in the transportation of E-3M2H by covalently bonding to it, forming an odor precursor (16). A previous study revealed that ApoD functions as one of the key transport proteins in the process of odor production and that ApoD is expressed at significantly higher levels in patients with osmidrosis compared with individuals without significant axillary odor (4).

The SNP of the *ABCC11* gene and the ApoD transfer protein have previously been associated with axillary odor; however, whether there is a correlation between the 538 G>A SNP and the expression of *ApoD* remains to be elucidated (10). Therefore, the present study further investigated the association between the 538 G>A SNP of *ABCC11* and the mRNA expression levels of *ApoD* in the apocrine glands. As the G allele is dominant, the GG and GA genotypes are phenotypically identical (17). The patients were divided into two groups according to genotype: Group I included individuals with GG or GA genotypes and Group II includedindividuals with the AA genotype. It has been reported that *ApoD* mRNA is exclusively expressed in the apocrine glands of the axillary tissues (9); therefore, the *ApoD* mRNA detected from axillary subcutaneous tissues accurately represents the expression of *ApoD* by the apocrine glands. Additionally, other possible influencing factors, including gender, age, BMI, diet, odor severity and family history, were also evaluated. A previous study suggested that *ApoD* may be a downstream target of regulating androgen receptor signals (18). The apocrine glands are direct targets of steroid hormone signaling and steroid hormones may therefore affect their activity (19). *In vivo*, steroid hormone levels vary amongst individuals at various time-points, particularly between genders. Therefore, fluctuation in axillary odor is frequently observed, emerging during teenage years, remaining throughout young adulthood and gradually declining to absence in individuals over 50 years old. However, steroid hormones have been observed to affect the quantity and distribution of body fat and have a negative correlation with obesity (20,21). For these reasons, individuals requiring surgery and aged between 15 and 45 years old were selected, and variations in age, gender and BMI were analyzed. Family history may reflect the heredity characteristic of osmidrosis and introduce an additional genetic interference factor, therefore, family history was also analyzed for its potential association with the *ABCC11* gene. In addition, dietary intake, particularly red meat, may alter the type and quantity of axillary fatty acid secretion in a short period and lead to a change in odor severity (5). Therefore, these data were analyzed and their potential effects on the experimental findings were evaluated. Significant differences were detected in gender, family

history and severity of odor, but not in age, BMI or dietary intake between the two groups. The variations in gender between the groups may have been due to men's ignorance of mild odor, resulting in the male subjects having a moderate or severe odor, which led to a selection bias. The differences in family history provided evidence for the genetic relevance of osmidrosis, which was clear in the results from patients with the G allele. The quantitative analysis demonstrated that the mRNA expression of *ApoD* was higher in patients with the G allele at 538 G>A compared with those without the G allele, suggesting that this SNP may affect the expression of *ApoD*. These results suggested that an SNP leading to a G180R substitution in the corresponding protein may decrease the transcription of *ApoD* through an unknown complex pathway.

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