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ORIGINAL ARTICLE

A Functional ABCC11 Allele Is Essential in the Biochemical Formation of Human Axillary Odor

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The characteristic human axillary odor is formed by bacterial action on odor precursors that originate from apocrine sweat glands. Caucasians and Africans possess a strong axillary odor ,whereas many Asians have only a faint acidic odor. In this study, we provide evidence that the gene *ABCC11* (*MRP8*), which encodes an apical efflux pump, is crucial for the formation of the characteristic axillary odor and that a single-nucleotide polymorphism (SNP) 538G→A, which is prominent among Asian people, leads to a nearly complete loss of the typical odor components in axillary sweat. The secretion of amino-acid conjugates of human-specific odorants is abolished in homozygotic carriers of the SNP, and steroidal odorants and their putative precursors are significantly reduced. Moreover, we show that ABCC11 is expressed and localized in apocrine sweat glands. These data point to a key function of ABCC11 in the secretion of odorants and their precursors from apocrine sweat glands. SNP 538G→A, which also determines human earwax type, is present on an extended haplotype, which has reached >95% frequency in certain populations in recent human evolution. A strong positive selection in mate choice for low-odorant partners with a dysfunctional *ABCC11* gene seems a plausible explanation for this striking frequency of a loss-of-function allele.

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INTRODUCTION

The human armpit has been named a ''scent organ'' as it has a central function in human body odor formation. Apocrine sweat glands in the axillary region secrete a variety of odor precursors that are transformed into volatile odoriferous substances by bacterial enzymes on the skin surface (Natsch *et al.*, 2003, 2005, 2006). The main contributors to axillary odor are (i) unsaturated or hydroxylated branched fatty acids with (E)-3-methyl-2-hexenoic acid (3M2H) and 3-hydroxy-3-methyl-hexanoic acid (HMHA) as key components, (ii) sulfanylalkanols, particularly 3-methyl-3-sulfanylhexan-1-ol (3M3SH) (Hasegawa *et al.*, 2004; Natsch *et al.*, 2004; Troccaz *et al.*, 2004), and (iii) the odoriferous steroids, 5α-androst-16-en-3-one and 5α-androst-16-en-3α-ol, which exhibit a urine- or musk-like smell (Bird and Gower, 1981). Although the precursors of odorant acids have been shown to be glutamine conjugates that are cleaved specifically by a bacterial N_{α}-acyl-glutamine-aminoacylase from *Corynebacterium striatum* A×20, sulfanylalkanols are secreted from apocrine sweat glands as cysteine–(S) or cysteine–glycine–(S) conjugates (Starkenmann *et al.*, 2005; Emter and Natsch, 2008), and are set free by the sequential action of a bacterial dipeptidase, tpdA, and a cysteine β-lyase, which have been cloned from the same bacterial strain (Emter and Natsch, 2008).

Apocrine sweat contains relatively high amounts of proteins (Froebe *et al.*, 1990; Jacoby *et al.*, 2004) and nonodoriferous steroids, for example, dehydroepiandrosterone (DHEA), androsterone, testosterone, and their corresponding sulfates (Toth and Faredin, 1985). It has been suggested by several authors that the two odoriferous steroids, 5α-androst-16-en-3-one and 5α-androst-16-en-3α-ol, are formed from nonodoriferous steroid precursors as a result of bacterial metabolism (Labows *et al.*, 1979; Gower *et al.*, 1997; Decreau *et al.*, 2003).

In contrast to the well-characterized influence of skin microflora and bacterial enzymes on axillary odor formation, only little is known about the fundamental metabolic and transport processes occurring in the apocrine sweat gland leading to the secretion of odorant precursor molecules. Recently, Yoshiura *et al.* (2006) showed that gene *ABCC11 (MRP8),* encoding an ATP-driven efflux pump, has a key role in the function of ceruminous apocrine glands that are localized in the auditory canal. The authors reported that a SNP, 538G→A, in *ABCC11*, leading to a G180R substitution in the corresponding protein, provokes a dry and white earwax phenotype, which is predominant among East Asians (80–95%) and rare among European and African populations (0–3%), which normally have a wet and yellow earwax phenotype. Quite recently, Toyoda *et al.* (2009) showed that

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Abbreviations: ABCC11, ATP-binding cassette, sub-family C, member 11; DHEA, dehydroepiandrosterone; DHEAS, dehydroepiandrosterone sulfate; HMHA, 3-hydroxy-3-methyl-hexanoic acid; med. conc., median concentration; 3M2H, (E)-3-methyl-2-hexenoic acid; 3M3SH, 3-methyl-3-sulfanylhexan-1-ol

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the nucleotide polymorphism affects *N*-linked glycosylation of ABCC11, resulting in proteasomal degradation of the variant protein. The SNP in *ABCC11* is unique, as it has been described as the only human SNP determining a visible genetic trait. Furthermore, it is present on an extended haplotype, which seems to have risen rapidly in frequency from a recent founder (Yoshiura *et al.*, 2006), indicating a strong positive selection for this (or the linked) SNP. Interestingly, it was previously reported that the characteristics of earwax are interconnected with the strength of axillary odor, whereby wet earwax is accompanied by a strong axillary odor and dry earwax goes along with a missing axillary odor (Adachi, 1937). The connection of earwax and axillary odor can be explained by the close relationship between ceruminous and apocrine sweat glands, both belonging to apocrine-type glands that share many histological and functional features (Stoeckelhuber *et al.*, 2006). Recently, the ABCC11 genotype was associated with colostrum secretion as well as osmidrosis (Miura *et al.*, 2007; Toyoda *et al.*, 2009).

ABCC11 is known to transport a variety of lipophilic anions (Chen *et al.*, 2005; Kruh *et al.*, 2007), including cyclic nucleotides, estradiol 17-β-D-glucuronide, steroid sulfates such as dehydroepiandrosterone sulfate (DHEAS), and monoanionic bile acids. As many of these metabolites share similarities with diverse components of axillary apocrine sweat, we hypothesized that ABCC11 may have an important role in the transport processes of apocrine odor molecules and that the above mentioned SNP in the *ABCC11* gene may lead to a loss of the secretion of metabolites that normally are essential for the formation of typical axillary odor. To verify this hypothesis, we carried out a detailed chemical analysis of axillary sweat samples from 25 subjects of different *ABCC11* genotypes. Moreover, we demonstrated the expression and localization of ABCC11 in human apocrine sweat glands.

RESULTS

ABCC11 genotyping and sweat sampling

The *ABCC11* genotype (SNP at 538, G→A) of 25 volunteers was analyzed (Table 1). Among the Asian panelists, most of whom originated from the Philippines, China, and Korea, 11 AA homozygotes, 5 AG heterozygotes, and 2 GG homozygotes were identified. Five of the seven tested Caucasian volunteers originating from Germany and Switzerland were classified as GG homozygotes, whereas only two showed an AG genotype.

All genotyped panelists were invited for axillary sweat sampling during a 30-min period of physical exercise on an exercise machine, during which time they wore sweat-absorbing cotton pads in their armpits. For each subject, between 0.39 g and 4.05 g of axillary sweat was sampled and extracted. The median sweat amounts collected for genotypes AA (1.28 g), AG (0.9 g), and GG (0.71 g) were not significantly different.

To investigate whether there are differences with regard to axillary microflora between different *ABCC11* genotypes, the diversity and number of axillary bacteria were determined. Superficial skin scraping samples from the axillary region were taken from 10 subjects of AA and from 7 subjects of AG/GG genotype, from which bacterial diversity was analyzed using the culture-independent DNA fingerprint method of single-strand conformation polymorphism (SSCP) (Schwieger and Tebbe, 1998) profiling (AMODIA Bioservices GmbH, Braunschweig, Germany). The axillary flora of all samples was found to be a mixture of mainly *Staphylococci*, *Corynebacteria,* and *Propionibacteria*. However, no statistical significance could be observed between subjects of different genotypes (data not shown). Furthermore, the total number of bacteria in the scraping samples was assessed using agar plates (COST, aerobic cultivation; Schaedler, anaerobic cultivation), which showed no significant differences either (data not shown).

Quantitative analysis of axillary odorant precursors by liquid chromatography-mass spectrometry

The glutamine conjugates, 3M2H–Gln and HMHA–Gln, as well as the Cys–Gly–(S) conjugates of 3M3SH (for structures, see Figure 1), have been described as precursors for key body odorants earlier (Natsch *et al.*, 2003, 2006; Starkenmann *et al.*, 2005; Emter and Natsch, 2008). The amount of these three precursors in axillary sweat from different *ABCC11* genotypes was analyzed by liquid chromatography-mass spectrometry (LC-MS) from the aqueous fraction of sweat samples (Table 1). All three conjugates were below detection limit in the samples of all panelists with the AA genotype. By contrast, in all axillary sweat samples from AG heterozygotes and GG homozygotes, the Gln conjugates, 3M2H–Gln (median amount AG: 0.17 μmol, median amount GG: 0.16 μmol) and HMHA–Gln (median amount AG: 1.18 μmol, median amount GG: 0.85 μmol), were detected. The Cys–Gly–(S) conjugate of 3M3SH was above the detection limit for 12 of the 14 AG and GG genotypes, although below the quantification limit for most samples. These data indicate that ABCC11 is essential for the secretion of amino-acid conjugates of relevant axillary odorants.

Quantitative analysis of enzyme-released odorants from axillary sweat samples

To further investigate the impact of different *ABCC11* genotypes on human axillary odor, the aqueous fractions of all axillary sweat samples were digested using enzymes N_α-acyl-glutamine-aminoacylase, cysteine β-lyase, and dipeptidase, tpdA, from the skin isolate, *Corynebacterium striatum* A×20, to release the axillary odorants from their nonodoriferous conjugates (Table 2). Subsequently, odorants were analyzed using two-dimensional GC coupled with time-of-flight mass spectrometry (GC × GC-ToF MS). The branched acids released by N_α-acyl-glutamine aminoacylase were present below the detection limit (analyte B3) or at very low levels (analytes A2 and E1) in genotype AA. These results are consistent with the fact that the Gln conjugates of these acids were below detection limit in the LC-MS analysis (Table 1). The same pattern as for branched acids could be shown for the straight acid C3, but the difference was less pronounced for the minor analyte C1. Detailed results of a larger series of enzyme-released acids, most of which were

Table 1. Amino-acid conjugates of key human body odorants in sweat samples of panelists with different genotypes, determined by LC-MS

Genotype *ABCC11*	Sex	Age	Ethnic population	Net weight sweat (g)/2 pads	Secreted amino-acid conjugates (μmol/2 pads)		
					HMHA–Gln	3M2H–Gln	Cys–Gly conjugate of 3M3SH
AA	M	29	Filipino	1.58	ND[1]	ND	ND
AA	F	43	Chinese	1.28	ND	ND	ND
AA	M	43	Filipino	1.18	ND	ND	ND
AA	F	35	Korean	1.11	ND	ND	ND
AA	F	27	Chinese	2.05	ND	ND	ND
AA	F	47	Filipino	1.10	ND	ND	ND
AA	F	50	Filipino	0.72	ND	ND	ND
AA	F	33	Filipino	2.02	ND	ND	ND
AA	M	28	Hong Kong Chinese	0.60	ND	ND	ND
AA	F	40	Filipino	1.31	ND	ND	ND
AA	F	40	Filipino	1.67	ND	ND	ND
MEDIAN AA				1.28			
AG	F	31	Filipino	1.47	1.23	0.17	Detectable, <0.03 μmol
AG	F	38	Filipino	0.84	1.58	0.23	0.041
AG	M	32	Filipino	0.83	0.06	Detectable, <0.03 μmol	ND
AG	M	37	Filipino	0.59	2.71	0.40	Detectable, <0.03 μmol
AG	F	25	Thai	0.90	0.89	0.14	Detectable, <0.03 μmol
AG	M	31	German	1.42	1.18	0.18	0.045
AG	F	25	German	1.64	0.54	0.10	Detectable, <0.03 μmol
MEDIAN AG				0.90	1.180	0.170	
GG	F	45	Filipino	1.74	0.77	0.13	Detectable, <0.03 μmol
GG	F	45	Filipino	0.39	0.75	0.11	Detectable, <0.03 μmol
GG	F	28	German	0.71	1.30	0.19	0.041
GG	F	33	German	1.23	1.12	0.16	0.038
GG	M	25	German	0.45	2.65	0.43	0.051
GG	F	35	German	0.68	0.34	0.09	Detectable, <0.03 μmol
GG	M	39	Swiss	4.05	0.85	0.18	ND
MEDIAN GG				0.71	0.85	0.160	

HMHA, 3-hydroxy-3-methyl-hexanoic acid; LC-MS, liquid chromatography-mass spectrometry; 3M2H; (E)-3-methyl-2-hexenoic acid; 3M3SH: 3-methyl-3-sulfanylhexan-1-ol.
[1]ND indicates that no detectable peak is found on the $[M+H]^+$ ion trace of the selected analyte at the correct retention time.
[2]A detectable peak at the correct retention time is found on $[M+H]^+$ ion trace of the selected analyte, but below quantification limit.

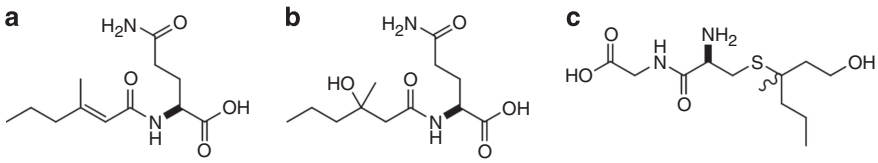
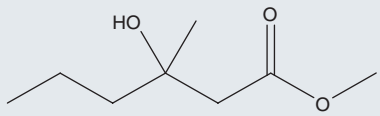
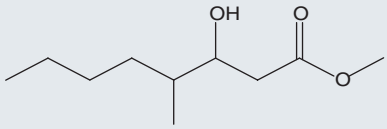
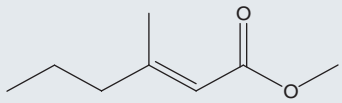
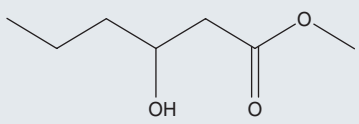
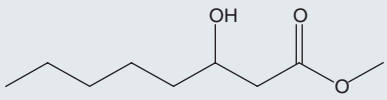


Figure 1. Structures of the key known human body odor precursors. (**a**) N_α-3-methyl-2-hexenoyl-glutamine (3M2H–Gln). (**b**) N_α-3-methyl-3-hydroxy-hexanoyl-glutamine (HMHA–Gln). (**c**) Cys–Gly conjugate of 3-methyl-3-sulfanyl-hexanol (3M3SH).

under detection level for AA homozygotes, are shown in the supplementary information (Supplementary Table S1). The enzyme-released thiol compound, 3M3SH, was found in six of eight GG panelists and in one of six AG panelists, but it was not detectable in AA homozygotes (data not shown).

Table 2. Enzyme-released odorants in sweat samples of panelists with different genotypes, determined by GC × GC-ToF MS

Identifier	Structure	Name	µg analyte/2 pads (median per genotype) AA	AG	GG	GG and AG	M-W *U* test (*P*-values) GG and AG *versus* AA
A2		3-hydroxy-3-methylhexanoic acid methyl ester	0.14	44.70	45.82	45.26	0.0000
B3		3-hydroxy-4-methyloctanoic acid methyl ester	0.00	1.84	0.69	0.75	0.0000
E1		(E)-3-methyl-2-hexenoic acid methyl ester	0.02	>>2.8[1]	>>2.8	>>2.8	0.0000
C1		3-hydroxyhexanoic acid methyl ester	0.03	0.08	0.13	0.12	0.035
C3		3-hydroxyoctanoic acid methyl ester	0.09	1.29	1.98	1.77	0.0001

GC × GC-ToF MS, two-dimensional GC coupled with time-of-flight mass spectrometry.
[1]This analyte was above the linear range for most of the AG and GG panelists.

Quantitative analysis of free acids in axillary sweat samples

Carboxylic acids, which are present in sweat in an unconjugated form, were directly determined from the acidic fraction of axillary sweat extracts from subjects of different *ABCC11* genotypes by gas chromatography-mass spectrometry (GC-MS) (Table 3a). In Table 3b, the detected amounts of different acids were normalized to the amounts of hexadecanoic acid, which is known to be one of the main components of *stratum corneum* lipids. Interestingly, the levels of long-chain fatty acids, hexadecanoic acid, octadecanoic acid, and linolic acid, are similar between different *ABCC11* genotypes (*P*-values > 0.3). The same is true for the short straight-chain fatty acids, butyric acid, hexanoic acid, and octanoic acid (*P*-values > 0.1), which are odoriferous, although to a much lesser extent than branched acids. By contrast, the levels of the odorous branched acids, 3-methyl butyric acid (isovaleric acid) and 2-methyl butyric acid, are significantly reduced in sweat samples from AA homozygotes compared with those from AG and GG genotypes (*P*-values < 0.005).

Analysis of steroid and protein concentration in axillary sweat samples

The content of the odoriferous steroids, 5α-androst-16-en-3-one and 5α-androst-16-en-3α-ol, in axillary sweat was analyzed in the aqueous fraction of extracted sweat samples by ELISA (Table 4) using a rabbit polyclonal antibody developed against androstenone-3-(*O*-carboxymethyl)oxime, which was coupled to keyhole limpet hemocyanin to induce immunogenicity of the relatively small steroid. As protein coupling involved the oxygen atom at position C3 of the steroid, the antibody was unable to distinguish between 5α-androst-16-en-3-one and 5α-androst-16-en-3α-ol. Although axillary sweat from GG homozygotes and AG heterozygotes showed high median concentrations of the steroids detectable by this ELISA analysis (median concentration AG: 1070.18 ng/ml sweat, med. conc. GG: 1371.26 ng/ml sweat), the concentrations were significantly reduced in AA homozygotes (med. conc. AA: 302.16 ng/ml sweat; *P*-value AA versus AG and GG: $P = 0.000$).

Table 3. (a) Free acids in sweat samples of panelists with different genotypes, absolute values (b) Free acids in sweat samples of panelists with different genotypes, normalized values

(a)

	µg/2 pads (median per genotype)				Comparison between genotypes (*P*-values)			
					Kruskal–Wallis test			M–W *U* test
	AA	AG	GG	AG and GG	AA *versus* AG	AA *versus* GG	AG *versus* GG	GG and AG *versus* AA
Butyric acid	0.61	0.99	1.27	1.03	0.684	0.221	964	0.338
3-methyl-butyric acid	0.08	0.23	0.76	0.34	0.113	**0.002**	0.189	**0.005**
2-methyl-butyric acid	0.23	2.60	12.91	5.64	0.021	**0.000**	0.094	**0.000**
Hexanoic acid	1.37	0.85	1.07	0.96	0.390	0.964	0.821	0.603
Octanoic acid	1.38	0.77	1.42	1.04	0.221	0.964	0.684	0.460
Hexadecanoic acid	194.13	138.51	385.43	151.59	0.556	0.258	0.497	0.763
Octadecanoic acid	90.12	45.85	200.09	70.48	0.556	0.298	0.189	0.805
Linoleic acid	18.41	11.93	42.80	13.11	0.618	0.342	0.618	0.805

(b)

	% of Hexadecanoic acid (median per genotype)				Comparison between genotypes (*P*-values)			
					Kruskal–Wallis test			M–W *U* test
	AA	AG	GG	AG and GG	AA *versus* AG	AA *versus* GG	AG *versus* GG	GG and AG *versus* AA
Butyric acid	0.38	0.61	0.35	0.45	0.094	0.751	0.033	0.427
3-Me-Butyric acid	0.07	0.18	0.22	0.22	**0.003**	**0.001**	0.160	**0.000**
2-Me-Butyric acid	0.24	2.47	4.21	3.50	**0.001**	**0.000**	0.052	**0.000**
Hexanoic acid	0.85	0.59	0.52	0.58	0.618	0.063	0.556	0.1627
Octanoic acid	0.88	0.54	0.49	0.54	0.258	0.033	0.497	0.052
Octadecanoic acid	44.95	45.33	53.30	49.55	0.751	0.094	0.258	0.239
Linoleic acid	11.01	11.20	10.70	10.71	0.497	0.342	0.964	0.891

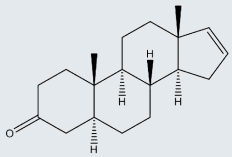
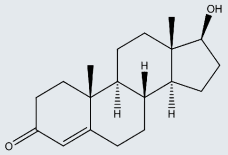
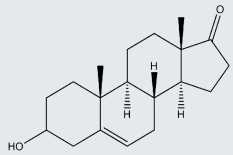
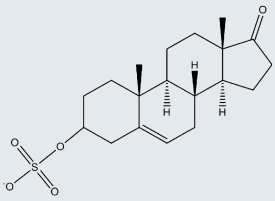
To investigate whether the *ABCC11* genotype also has an impact on metabolites of the apocrine sweat gland that are non-odoriferous, we quantified the amounts of steroids DHEA and testosterone from the organic fraction and sulfated dehydroepiandrosterone (DHEAS) from the aqueous fraction of axillary sweat samples by ELISA (Table 4). Moreover, we analyzed the protein concentration in the aqueous fraction of sweat samples (Table 5). For both DHEA and DHEAS, we found significantly lower concentrations in samples from AA homozygotes in comparison with samples from AG and GG genotypes (DHEA: med. conc. AA: 2.04 ng/ml sweat, med. conc. AG: 94.34 ng/ml sweat, med. conc. GG: 131.70 ng/ml sweat; *P*-value AA versus AG and GG: $P=0.000$; DHEAS: med. conc. AA: 1.85 µg/ml sweat, med. conc. AG: 41.36 µg/ml sweat, med. conc. GG: 14.99 µg/ml sweat; *P*-value AA versus AG and GG $P=0.000$). The same could be shown for protein concentrations (med. conc. AA: 276.79 µg/ml sweat, med. conc. AG: 825.87 µg/ml sweat, med. conc. GG: 1041.31 µg/ml sweat; *P*-value AA versus AG and GG: $P=0.018$; Table 5). By contrast, testosterone levels were found to be similar in all three genotypes (med. conc. AA: 0.24 ng/ml sweat, med. conc. AG: 0.33 ng/ml sweat, med. conc. GG: 0.46 ng/ml sweat; *P*-value AA versus AG and GG: $P=0.057$).

Expression and localization of ABCC11 in apocrine sweat glands

The expression of the ABCC11 transcript was investigated by RT–PCR analysis of sweat gland curettage samples from the armpit of hyperhidrotic patients (Figure 2), which are known to be rich in apocrine sweat glands (Bechara *et al.*, 2008). As control tissue, we used face skin from the upper eyelid (below the eyebrow). Expression of *ABCC11* on transcript level was present only in curettage samples but could not be detected in control samples from eyelid skin. In contrast, expression of the housekeeping gene *GAPDH* was present in both sample types, suggesting that *ABCC11* expression is specific in tissue containing apocrine sweat glands.

Similar results for *ABCC11* expression were obtained by cDNA microarray analyses (Agilent Whole Human Genome Oligo Microarrays, Miltenyi Biotec, Bergisch Gladbach, Germany) of laser-microdissected apocrine sweat glands using eccrine sweat glands as control tissue for comparison of gene expression. In apocrine gland tissue, *ABCC11*

Table 4. Steroid concentrations in sweat of panelists with different genotypes, analyzed by ELISA

Structure	Name	ng analyte/ml sweat (median per genotype) AA	AG	GG	Kruskal–Wallis test (*P*-value) AA *versus* AG	AA *versus* GG	AG *versus* GG	M–W *U* (*P*-value) GG and AG *versus* AA
	5α-androst-16-en-3-one[3]	302.16	1,070.18	1,371.26	0.016	0.003	1.000	0.000
	Testosterone	0.24	0.33	0.46	0.734[1]	0.067[1]	0.260[1]	0.057[2]
	Dehydroepiandrosterone	2.04	94.34	131.70	0.001	0.001	1.000	0.000
		µg analyte/ml sweat (median per genotype)						
	Dehydroepiandrosterone sulfate	1.85	41.36	14.99	0.002	0.003	1.000	0.000

[1]Due to normal distribution, a Tukey's honest significance test for unequal sample sizes was employed.
[2]Student's *t*-test was applied.
[3]Used antibody is not able to distinguish between 5α-androst-16-en-3-one and 5α-androst-16-en-3α-ol.

Table 5. Protein concentrations in axillary sweat samples of panelists with different genotypes

µg protein/ml sweat (median per genotype)			
AA	AG	GG	AG and GG
276.79	825.87	1041.31	847.66

Kruskal–Wallis test (*P*-value)			M–W *U* (*P*-value)
AA *versus* AG	AA *versus* GG	AG *versus* GG	AA *versus* AG and GG
0.037	0.021	1.000	0.018

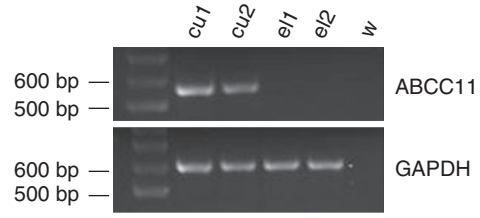


Figure 2. Reverse transcriptase PCR of *ABCC11* in curettage of sweat glands. A specific *ABCC11* PCR product of 556 bp was detected in curettage samples of human sweat glands (cu1, cu2), whereas in samples from eyelid skin (el1, el2) no PCR product was amplified. *GAPDH* expression (amplified PCR product: 597 bp) was detected in both, curettage and eyelid skin samples. w: water control.

expression was 1.63-fold above a common reference that was computed by creating an artificial pool of gene expression composed of all 22 individual profiles (apocrine and eccrine samples from 11 donors, data not shown). In eccrine gland tissue, *ABCC11* expression was downregulated 21.40-fold compared with the above-mentioned common reference

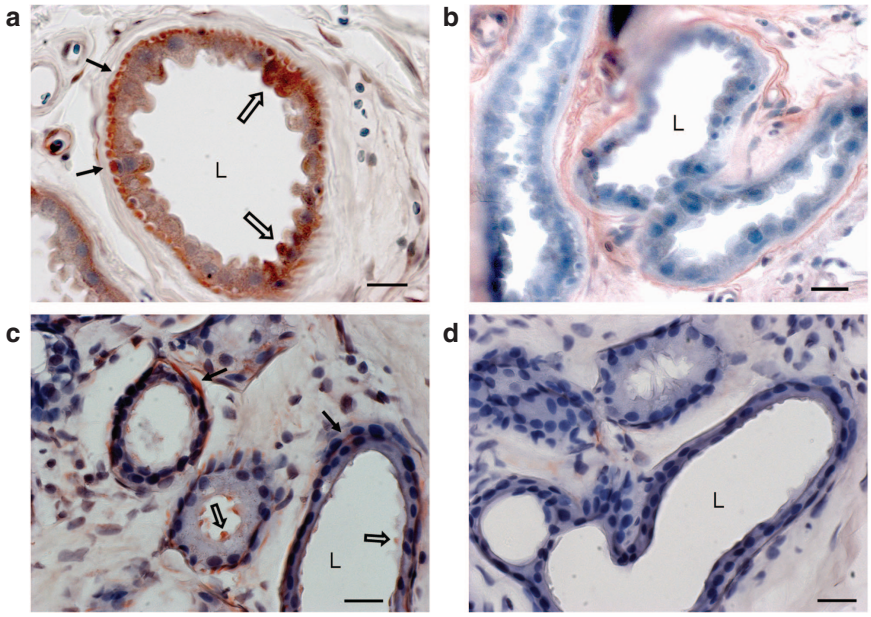


Figure 3. Immunolocalization of ABCC11 protein in apocrine sweat glands of different ABCC11 genotypes. (**a**) GG genotype. Even ABCC11 staining was detected in myoepithelial cells (→). Moreover, a strong ABCC11 signal was found in single secretory cells (⇒). Scale bar = 20 µm. (**b**) GG genotype. Negative control without primary antibody. Scale bar = 20 µm. (**c**) AA genotype. ABCC11 staining is visible in myoepithelial cells (→) and single secretory cells (⇒). Scale bar = 20 µm. (**d**) AA genotype. Negative control without primary antibody. Scale bar = 20 µm. Blue signal: nuclei stained by Mayer's hematoxylin; L: lumen of apocrine sweat gland; reddish brown signal: specific ABCC11 staining.

(*ABCC11* expression differences between apocrine and eccrine gland tissue as determined by Pavlidis template matching: *P*-value = 7.5×10^{-9}).

The localization of ABCC11 protein in apocrine sweat glands was analyzed by immunohistochemistry of cryosections from axillary skin samples of a GG homozygote using a polyclonal antibody against amino acids 929–1144 of the human ABCC11 protein. Expression of ABCC11 could be shown within the layer of myoepithelial cells surrounding the secretory cells of the apocrine coil (Figure 3a). Moreover, a strong ABCC11 expression was found in single secretory cells of the apocrine sweat gland, whereas a slight, diffuse signal was apparent in the apical region of several secretory cells. In cryosections of axillary skin from AA homozygotes (Figure 3c), the ABCC11 signal was concentrated in the same parts of apocrine glands as those in GG homozygotes, indicating that the nonfunctional ABCC11 protein is still translocated to its site of action. No staining was detected in negative controls carried out by the omission of the primary antibody (Figure 3b and d). It is noteworthy that apocrine sweat glands in AA homozygotes showed a slightly different morphology compared with those in GG homozygotes (Figure 3).

DISCUSSION

ABCC11 is crucial for the secretion of odorants and their precursors from the apocrine sweat gland

The impact of the *ABCC11* genotype on the characteristics of earwax and colostrum was shown previously (Yoshiura *et al.*, 2006; Miura *et al.*, 2007). By analyzing axillary sweat samples from different *ABCC11* genotypes, we provide evidence that ABCC11 is directly required for the secretion of characteristic components contributing to axillary odor formation. These data indicate that ABCC11 is essential for the secretion of the amino-acid conjugates 3M2H–Gln, HMHA—Gln, and Cys–Gly–(S) 3M3SH (Table 1), which are precursors of the key body odorants, 3M2H, HMHA, and 3M3SH. After enzymatic release from their precursor molecules, all analyzed branched chain odorants were significantly reduced in panelists of the AA genotype (Table 2, Supplementary Table S1). Interestingly, this effect was less pronounced for the aminoacylase-released straight acids, 3-hydroxyhexanoic acid and 3-hydroxyheptanoic acid, which are known as general β-oxidation metabolites and were not exclusively described as axillary metabolites.

The branched acids, 3-methyl butyric acid and 2-methyl butyric acid, were also clearly reduced in the AA genotype (Table 3), indicating that ABCC11 has a high impact on the secretion of compounds originating from the leucine/isoleucine degradation pathway in apocrine sweat glands that seem to leave the gland as free acids. In contrast, the long and the short straight-chain acids are not affected by the *ABCC11* genotype. They might be secreted by different glands, by an ABCC11-independent process, or might even be derived from β-oxidation or bacterial degradation of skin or sebum lipids. It is very likely that these aliphatic acids are responsible for a faint sour acidic axillary odor that has been described for the majority of Japanese volunteers previously (Hasegawa *et al.*, 2004).

The concentrations of the odoriferous steroids, 5α-androst-16-en-3-one and 5α-androst-16-en-3α-ol, detected by ELISA assay, were significantly reduced in panelists of the AA genotype compared with genotypes AG and GG (Table 4).

Furthermore, it should be noted that the nonodoriferous androgen, DHEA, and its sulfated derivative, DHEAS, which are believed to be precursors of the bacterial metabolism leading to the formation of 5α-androst-16-en-3-one in axillary sweat (Labows *et al.*, 1979; Gower *et al.*, 1997), were shown to be reduced in AA genotypes. Several authors have shown that a functional ABCC11 protein is able to transport DHEAS (Chen *et al.*, 2005; Bortfeld *et al.*, 2006; Kruh *et al.*, 2007). It is therefore plausible that a nonfunctional ABCC11 transporter leads to a strong decrease in DHEAS and DHEA in axillary apocrine sweat, which in turn might decrease bacterial production of odoriferous steroids. In contrast to DHEA and DHEAS, testosterone concentrations were not significantly reduced in the axillary sweat of AA panelists, suggesting that ABCC11 has no impact on the transport and metabolism of sex steroids.

Besides a strong reduction in axillary odor compounds in sweat samples of AA panelists, the nonfunctional *ABCC11* allele leads to a decrease in protein concentration in axillary sweat (Table 5). This might explain the results recently published by Jacoby *et al.* (2004) on apocrine secretion odor-binding protein 2, one of the proteinaceous components of apocrine sweat. This protein was shown to be clearly reduced in axillary sweat samples of Chinese panelists (which might have been of the AA genotype), but not in panelists of European, African, and Pakistani descent.

According to literature, ABCC11 possesses broad substrate specificity for different lipophilic compounds (Guo *et al.*, 2003; Chen *et al.*, 2005; Kruh *et al.*, 2007), suggesting that ABCC11 may directly transport lipohilic odorants and amphiphilic odorant precursors in apocrine sweat glands. However, it remains to be clarified whether protein concentrations are affected directly by ABCC11 transport or by a regulatory function of ABCC11 that finally influences protein metabolism in the apocrine sweat gland.

Axillary malodor formation is closely linked to the activity of axillary skin bacteria, mainly *Staphylococci* and *Corynebacteria* (Leyden *et al.*, 1981; Natsch *et al.*, 2003, 2005, 2006; Taylor *et al.*, 2003; Troccaz *et al.*, 2004). However, the marked reduction in odoriferous components in the axillary sweat of AA haplotypes seems to be independent from the axillary microflora, which could be shown to be comparable with one of the subjects with AG and GG genotypes regarding the amount and diversity of skin bacteria.

ABCC11 is localized in the axillary apocrine sweat gland

The data from RT–PCR, gene–chip analysis, and immunohistological stainings demonstrate that ABCC11 is expressed in axillary apocrine sweat glands of panelists of wild type *ABCC11* (GG) both at the transcript and at the protein level (Figure 2 and Figure 3a). Interestingly, in immunohistological stainings, antibody signal was found in both myoepithelial cells and secretory cells. Expression of ABCC11 in the secretory cells of apocrine sweat glands could indicate a direct function of this transport protein in the process of apocrine secretion and is consistent with the data on apocrine sweat compounds being strongly decreased in AA panelists. An often described, characteristic of the apocrine secretion process is the release of the apical cell pole (decapitation) during secretion (Aumuller *et al.*, 1999; Saga, 2001). However, little is known about the molecular mechanisms of apocrine secretion (Gesase and Satoh, 2003). A very recent publication on ABCC11 expression in cerumen glands proposed that ABCC11 is localized on membranes of intracellular granules and vacuoles, where it transports ceruminous materials for sequestration inside these compartments (Toyoda *et al.*, 2009). Interestingly, Bang *et al.* (1996) described apocrine sweat glands of normal Korean panelists as being atrophic and small, and without any signs of decapitation. As in Korea, a high percentage of the population carries the AA alleles of *ABCC11*, it is conceivable that a functional ABCC11 transport protein is essential not only for the transport of apocrine metabolites but also for the function and structure of apocrine sweat glands. Indeed, our microscopic analysis of the apocrine glands of an AA panelist indicated a slightly different morphology and a slightly smaller gland size, but with a detectable ABCC11 expression (Figure 3c). Thus, a dysfunctional ABCC11 secretion process may have a pleiotropic effect on apocrine glands at the morphological level as well.

The expression of ABCC11 in myoepithelial cells may suggest that this transport protein also has a role in the supply of substances for further metabolism from myoepithelial to secretory cells of the apocrine sweat gland.

Possible evolutionary implications of the ABCC11 mutation

Axillary odor components are believed to have a role in nonverbal communication between humans, primarily as alarm (Ackerl *et al.*, 2002; Pause *et al.*, 2004; Prehn *et al.*, 2006; Mujica-Parodi *et al.*, 2009) or sex pheromones (Cutler *et al.*, 1986; Pause, 2004; Saxton *et al.*, 2008). Moreover, signals with regard to individual MHC properties are supposed to be transmitted by axillary odor (Wedekind *et al.*, 1995; Wedekind and Furi, 1997; Wedekind and Penn, 2000; Havlicek and Roberts, 2009). According to Yoshiura *et al.* (2006), the described SNP in *ABCC11* in this study originated in northeast Asia and then spread rapidly especially in the Asia-Pacific region. The SNP is mainly present on a conserved extended haplotype, which has undergone little recombination, indicating a recent evolutionary origin from a single founder (Yoshiura *et al.*, 2006). Thus, a high selection pressure must have acted on this haplotype to reach >95% frequency in large human populations. This is especially astonishing, as it is a loss-of-function mutation in a gene that has also been shown to be expressed in several other tissues such as testis, liver, placenta, lung, kidney, brain, and axons (Yabuuchi *et al.*, 2001). Does this selective advantage result from the ''white earwax'' phenotype, the change in colostrum contents, the loss of body odor, or even an unknown phenotypic alteration due to loss of ABCC11 function in another tissue? Certainly, it is impossible to decide this question *post hoc*, yet one may speculate that in old Eastern cultures with a more pronounced tradition of cleanliness and personal hygiene as compared to old Western cultures (Benn, 2004), the ''low-odor-phenotype'' conferred by a dysfunctional *ABCC11*

allele was important in partner selection and gave a mating advantage to the carriers of the AA genotype. At least from today's perspective, with axillary odors being perceived as socially offensive in Western cultures as well, this explanation for this unique selection pressure seems plausible, and would be supported by our data proving a crucial function of a functional *ABCC11* allele in the biochemistry of body odor formation.

MATERIALS AND METHODS

Genotyping

Twenty-five volunteers (5 Asian males, 13 Asian females, 3 Caucasian males, 4 Caucasian females) aged 25–50 years were genotyped with a focus on position 538 of the gene *ABCC11*. All volunteers were informed about the aim and purpose of this study and were asked to give written consent. Buccal cells were collected by scraping 10 times over the oral mucosa of the volunteers. Genomic DNA was purified from the buccal cells using the Puregene Buccal Cell Core Kit A (Gentra Systems, Minneapolis, MN) according to the manufacturer's instructions. PCR amplification was carried out using primers ABCC11_for (5′-GGATTGAAAAAG CTTCAGTGCT-3′) and ABCC11_rev (5′-CTAAGTGCCAGGGACAT GGT-3′). The amplified 354-bp fragment was purified using the QIAquick PCR purification kit (Qiagen, Hilden, Germany) and was subsequently sequenced using primer ABCC11_for.

Sampling and extraction of axillary secretions

In preparation for sweat sampling, subjects had to abstain from using deodorant and antiperspirant products for 5 days and used neutral soap (Baktolin (pH 5.5), Bode Chemie, Hamburg) for washing their armpits. Axilla secretions of individual donors were sampled on cotton pads fixed to the axilla during a 30-minute physical exercise on an exercise machine. During physical exercise, blood pressure and pulse were controlled regularly. Pads were frozen immediately after collection and transported to the analysis lab on dry ice. The net weight of the axilla secretions was determined by weighing fresh pads and dry pads after extraction. The cotton pads from both armpits were split into two halves. Two half-pads (one from each armpit) were pooled in a falcon tube and were extracted with 25 ml 20% EtOH in H_2O for 20 minute. The solution was separated from the pads by centrifugation and was then extracted using 5 ml cyclohexane. The cyclohexane fraction was amended with 80 µl isopropanol and concentrated to a final volume of 80 µl under a N_2 stream. This sample was diluted in 500 µl H_2O for storage and subsequent ELISA analysis, and it is referred to as the ''organic fraction''.

The aqueous phase after cyclohexane extraction was lyophilized and re-suspended in 600 µl H_2O. This is referred to as the ''aqueous fraction'' and it was further subjected to protein determination, ELISA assays, LC-MS analysis and enzymatic digestion for release of odorants (see below).

To investigate free acids in the fresh sweat, another half-pad was separately extracted: It was soaked with 3.5 ml of HCl (20 mM) for 15 minute and then the liquid phase was separated by centrifugation. A total of 2 ml diethylether was then added to the same pad, and this solvent was centrifuged into the same tube. The sample was then brought to basic pH with 100 µl 1-M NaOH. The organic phase containing neutral and basic analytes was discarded and the aqueous phase was acidified with 200 µl 1-M HCl, saturated with NaCl and extracted with 1 ml diethylether. This organic phase containing the acidic analytes was separated, amended with 50 µl isopropanol, and concentrated to a final volume of 100 µl under a N_2 stream. This sample is referred to as the ''acidic fraction''.

Analysis for odorant precursors by LC-MS

The aqueous fraction was centrifuged and 5 µl of the supernatant was subjected to LC-MS analysis using the method described by Natsch *et al.* (2003). In brief, a Finnigan (ThermoFinnigan, San Jose, CA) LCQ mass spectrometer operated in the APCI positive mode and equipped with a Flux Rheos 2000 HPLC pump was used. HPLC separation was carried out on a C18 RP column modified for proteins and peptides (VYDAC, Hesperia, CA). A binary eluent consisting of water (A) and methanol (B) each containing 1% acetic acid (v/v) was applied at a flow rate of 300 µl/minute. A linear gradient was applied changing from 10 to 98% B within the 3.5-minute period starting 1 minute after injection, then isocratic at 98% B for 4.5 minute. Afterwards, the B content was lowered to 10% over 1 minute and the column re-equilibrated for 2 min. Calibration solutions of the synthetic samples of 3M2H–Gln (for synthesis, see Natsch *et al.*, 2003), HMHA–Gln (for synthesis, see Natsch *et al.*, 2003), and the Cys–Gly–(S) conjugates of 3M3SH were measured and used for calculating the linearity. Signal areas were integrated using the extracted mass chromatograms of the $[M+H]^+$ ions (275 HMHA–Gln; 257 for 3M2H–Gln and 293 for the Cys–Gly–(S) conjugate of 3M3SH).

Digestion of the aqueous fractions with recombinant enzymes from Corynebacteria

After LC-MS analysis, the pellet obtained from the aqueous fraction was re-suspended in the supernatant, and this suspension was used for further analysis. Part (200 µl) of this sample was treated with 1.6 µg of N_α-acyl-glutamine-aminoacylase (Natsch *et al.*, 2003), 1.6 µg of cysteine β-lyase (Natsch *et al.*, 2004), and 1.6 µg of dipeptidase tpdA (Emter and Natsch, 2008). These enzymes were all cloned from the axilla isolate *Corynebacterium striatum* A×20 and heterologuously expressed in *E. coli*. (Natsch *et al.*, 2003, 2004; Emter and Natsch, 2008), and their involvement in the release of human body odorants from secreted amino-acid conjugates has been verified previously (Natsch *et al.*, 2003, 2004; Emter and Natsch, 2008). After 4 h incubation at 36 °C to ensure complete hydrolysis of the conjugates, the samples were extracted with 30 mg NaCl, 17 µl HCl (1 M) and 100 µl methyl-*tert*-butyl-ether. The organic phase (80 µl) was transferred to a GC vial, 5 µl of a 2 M solution of (trimethylsilyl)-diazomethane (Fluka, Buchs, Switzerland) and 18 µl methanol were added, and the sample was heated to 40 °C for 30 minute to form methyl esters of the enzymatically released acids.

GC × GC-ToF MS analysis of enzyme-released odorants

The released thiols and the methyl esters of the released acids were then analyzed using GC × GC-ToF MS as described in detail recently (Kuhn and Natsch, 2008). Following modifications as compared with the previously used method were applied: sample volumes of 3 µl were injected in the pulsed splitless mode. The inlet pulse pressure was set to 65.3 psi for 4.6 minute. A capillary column of 30 m length × 0.25 mm innerdiameter, coated with 0.25 µm of VF-5

MS (5% phenyl-polysiloxane, 95% dimethyl-polysiloxane) was used for the first-dimension oven (Varian, Palo Alto, CA). Separations were effected using the following temperature program: 35 °C, isothermal for 5 minute, then 10 °C/minute to 120 °C, 5 °C/minute to 210 °C, then 30 °C/minute to 260 °C, isothermal for 8 minute. The modulation time was 5 seconds. The separate second-dimension oven was kept at the first-dimension oven temperature, whereas the modulator housing was kept 15 °C above the first-dimension oven temperature. Besides the key acids in axilla secretions, 3M2H and HMHA, the peaks of several other acids were integrated (Kuhn and Natsch, 2008).

GC-MS analysis of free acids in fresh axilla secretions

The ''acidic fraction'' was analyzed with conventional GC-MS analysis. A combination of a Hewlett-Packard (Paolo Alto, CA) 5890 II gas chromatograph and a Finnigan MAT SSQ 7000 mass spectrometer (70 eV EI mode, ion source temperature 180 °C) was applied. A capillary column of 30 m length × 0.25 mm inner diameter, coated with 0.25 μm of VF-WAXms (polyethylene glycol, Varian, Palo Alto, CA) was used. Sample volumes of 1.0 μl were injected in the splitless mode at an injector temperature of 230 °C. Temperature of the column oven was initially set to 35 °C for 3 minute and subsequently increased by 15 °C/minute to 50 °C, then by 5 °C/minute to 240 °C and then by 10 °C/minute to 250 °C and held for 60 min.

Quantitative analysis of steroids from axillary sweat by ELISA

Quantitative analysis of DHEA, DHEAS and testosterone was done using ready-to-use ELISAs according to the manufacturer's instructions (DHEA Luminescence Immunoassay, DHEA-S ELISA, Testosterone Saliva ELISA; IBL, Hamburg, Germany). Test samples for DHEA and testosterone were taken from the organic fraction of extracted axillary sweat samples and were tested in repeat determination (diluted in sample diluent, IBL, Hamburg, Germany; DHEA: 1:101, testosterone: 1:10). For DHEAS analysis, test samples were taken from the aqueous fraction (diluted 1:76 in Standard A, included in the kit, IBL, Hamburg, Germany).

For the concurrent analysis of 5α-androst-16-en-3-one and 5α-androst-16-en-3α-ol, a polyclonal rabbit antiserum against androstenone-3-(*O*-carboxymethyl)oxime-keyhole limpet hemocyanin was used. For competition, androstenone-3-(*O*-carboxymethyl)oxime–horseradish peroxidase was used. Antibody as well as enzyme conjugate were kindly provided by Dr Hubert Kalbacher (Eberhard Karls University of Tübingen, Germany). Cross-reactivity of the antibody against testosterone as well as dehydroepiandrosterone was excluded by competition experiments (data not shown). The 96-well microtiter plates were coated with protein A (1 μg/well). Subsequently, 100 μl of rabbit antiserum (diluted 1:500) was applied to each well. After several washing steps using 0.002% Tween 20 (Merck, Darmstadt, Germany), 50 μl androstenone enzyme conjugate (1 μg/ml) and 50 μl of either aqueous phase of extracted sweat samples (diluted 1:10 in deionized water) or 5α-androst-16-en-3-one standard (Sigma-Aldrich, Steinheim, Germany) were added to each well. Incubation was performed at 37 °C for two hours. After repeated washing, 100 μl of ABTS substrate solution (2,2′-azino-bis-(3-ethylbenzthiazoline-6-sulphonic acid; Serva, Heidelberg, Germany) was added. Absorption was measured at 405 nm using the photometer Safire (Tecan, Crailsheim, Germany).

Determination of protein concentrations in axillary sweat

Determination of protein concentrations from the aqueous fraction of axillary sweat samples, diluted 1:5 in deinonized water, was carried out using the BC Assay Protein Quantitation small kit (Interchim, San Pedro, CA) according to the manufacturer's instructions. Absorption was measured at 562 nm using microplate reader SpectraMAX 250 (Molecular Devices, Sunnyvale, CA).

Axillary punch biopsies and sample preparation

Axillary punch biopsies were taken from four volunteers that had been tested for their *ABCC11* genotype, before. Two volunteers had an AA genotype whereas the other two were GG homozygotes. Volunteers were informed about the aim and purpose of the study and asked to give written informed consent. The study was approved by the local medical ethics committee (Ethik ÄKHH, Hamburg, Germany, reference no. 2222, study no. 28406). Axillary skin samples were obtained by standard punch biopsies with a diameter of 4 mm, following local anaesthesia (Maeverin 1%, Delta Select, Pfullingen, Germany) and immediately stored on dry ice. Afterwards, samples that consisted of epidermis, dermis, and subcutaneous regions of the skin, were frozen by plunging into liquid nitrogen and stored therein until further processing. Each sample was mounted on a sample holder using embedding medium (Shandon Cryomatrix, Thermo Scientific, Waltham, MA, USA) by positioning on its edge to allow for longitudinal sectioning of the different skin regions simultaneously. Serial sections (8 μm) were cut using a cryostat (CM3050S, Leica, Wetzlar, Germany), mounted on glass microscope slides (Superfrost, Fisher Scientific, Schwerte, Germany), air-dried and fixed in acetone for 20 minute at −20 °C in preparation for staining.

Immunohistochemistry

Sections were rehydrated with Tris-buffered saline (TBS) for 15 minute at room temperature and endogenous peroxidase activity was blocked by treatment with 3% H_2O_2 for 15 minute. In addition, sections were incubated with avidin D, followed by biotin incubation for 15 minute to inhibit endogenous biotin binding (Avidin/Biotin Blockin Kit, Vector Laboratories, Burlingame, CA). Sections were incubated with 10% normal donkey serum (Jackson Immunoresearch Europe, Suffolk, UK), diluted in TBS for 20 minute to prevent nonspecific antibody binding. Immediately, sections were incubated with a polyclonal rabbit IgG primary antibody against amino acids 929–1144 of human ABCC11 (Santa Cruz, Santa Cruz, CA), diluted to 1:100 in TBS, for 18 hour at 4 °C in a humidity chamber. A donkey biotinylated secondary antibody (Jackson Immunoresearch Europe, Suffolk, UK), diluted to 1:200 in TBS, was applied to the slides and incubated for 45 minute at room temperature. Signal amplification was achieved using the avidin–biotin–horseradish peroxidase complex method (Vectastin ABC Kit, Vector Laboratories, Burlingame, CA), according to the manufacture's instruction. Visualization of the immunoreactive sites was carried out using 3-amino-9-ethylcarbazole (AEC Substrate-Chromogen, DakoCytomation, Glostrup, Denmark). Between all applications, sections were washed for 15 minute with TBS. The sections were counterstained using Mayer's hematoxylin (Sigma-Aldrich) for 8 minute, rehydrated, and coverslipped using aqueous mounting medium (CV-Ultra, Leica, Wetzlar, Germany).

Negative controls were carried out by omission of the primary antibody. Frozen tissue sections of human breast cancer (BioChain, Hayward, CA) were used as positive controls (data not shown).

RNA extraction and reverse transcriptase PCR

Curettage and upper eyelid (from below the eyebrow) tissue samples for RT–PCR derived from plastic surgery. Patients gave written informed consent to further usage of tissue samples for scientific purpose. Total RNA was isolated from either 70 mg of curettage or eyelid tissue using the RNeasy Fibrous Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RT–PCR analysis was carried out using a standard reversed two-step transcription PCR. A total of 750 ng of total RNA was used for reverse transcription using the High Capacity cDNA Reverse Kit (Applied Biosystems, Foster City, CA). One-tenth of the reaction volume was amplified by PCR. The following primers were used for amplification of *ABCC11* and *GAPDH* cDNAs: ABCC11_for: 5′-GGATTGAAAAAGCTTCAGTGCT-3′, ABCC11_rev_2: 5′-GTGTGATGCTGAGCCTTCAC-3′ (size of *ABCC11* PCR product: 556 bp), GAPDH_for: 5′-CCACCCATGGCAAATTCCATGGCA-3′, GAPDH_rev: 5′-TCTAGACGGCAGGCAGGTCAGGTCCACC-3′ (size of *GAPDH* PCR product: 597 bp).

Statistical analysis

A Shapiro–Wilk test was used to determine whether the different datasets were normally distributed or not. In case of normal distribution, a Tukey's honest significance test for unequal sample sizes was used to compare all three genotypes. A Student's *t*-test was applied for a two-group comparison (AA versus AG and GG). After normal distribution has been excluded, statistical analysis was done using the Kruskal-Wallis test or the Mann–Whitney *U* (M-W-U) test as non-parametric methods.

All studies involving participants were conducted according to Declaration of Helsinki Principles.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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