

Original Article

Association between ApoD expression level and the severity of axillary osmidrosis

Yingli Li^{1,2*}, Xiao Gu^{3*}, Chao Yang^{2*}, Zhen Zhao^{4,5}

¹Department of Plastic Surgery, The General Hospital of Jinan Military Command, Jinan, Shandong, China;

²Department of Plastic Surgery, Chang Hai Hospital, Second Military Medical University, Shanghai, China;

³Department of Obstetrics, Shandong Provincial Hospital Affiliated to Shandong University, Jinan, Shandong, China; ⁴Department of Otolaryngology Head and Neck Surgery, 303 Hospital of PLA, Nanning, Guangxi, China;

⁵Department of Otolaryngology Head and Neck Surgery, Chang Hai Hospital, Second Military Medical University, Shanghai, China. *Equal contributors.

Received October 26, 2016; Accepted October 27, 2016; Epub February 1, 2017; Published February 15, 2017

Abstract: Axillary osmidrosis (AO) is a clinical condition in which apocrine glands cause strong unpleasant axillary odor. Although apolipoprotein D (ApoD) has been identified as an axillary odorant binding protein, its association with AO is largely unknown. In this study, we enrolled 78 AO patients who underwent surgical treatment in our hospital between 2012 and 2015. Sixteen skin repair male patients were used as controls. We analyzed the secretion status of apocrine glands in axillary tissues by H&E staining and compared ApoD protein expression by immunohistochemistry (IHC). When compared with controls, the apocrine glands in AO group were active with cells projecting towards the lumen, forming thicker walls and smaller chambers. Much more extensive cytoplasmic brown pigmentary granules were observed in the apocrine gland cells in AO group. The average optical density and integrated optical density (IOD) in AO group was significantly higher compared with controls ($P < 0.05$), suggesting a significantly higher ApoD expression in AO group. We further divided the AO patients into mild, middle and severe subgroups, and compared their expression of ApoD mRNA. We found that severe subgroup had higher mean ApoD mRNA expression ($P < 0.05$) and higher proportion of high ApoD expression when compared with the other subgroups ($\chi^2 = 12.06$, $P < 0.05$), suggesting a correlation between ApoD expression and the severity of AO. Our data revealed that ApoD were highly expressed in the apocrine sweat glands in AO, which might play an important role in the pathogenesis of the disease.

Keywords: Axillary osmidrosis, apocrine sweat glands, ApoD, immunohistochemistry

Introduction

Axillary osmidrosis (AO) has become a benign disorder that causes functional and emotional problems in Asian patients [1-3]. Sometimes, surgery to remove the axillary gland is performed on demand in Asian countries [4-8].

Previous studies [9] have shown that the characteristic odor which arises in the human axillary region consists of volatile C6-C11 acids. The E-isomer of 3-methyl-2-hexenoic acid (3M-2H) is the dominant analytical component of the mixture. This acid, as well as several other components of the characteristic axillary odor, can be liberated from the odorless, aqueous soluble components of apocrine secretion by either saponification or bacteriolysis [10]. In other words, the human odor might be carried

to the skin surface bound to a water soluble precursor where it is liberated by axillary bacteria. Recent studies have identified that 3M2H was liberated from ASOB1 and ASOB2 [11, 12], with molecular weights of 45 and 26 kd, respectively. ASOB2 is identical with ApoD in amino acid sequences [13]. However, its pattern of glycosylation differs from that of ApoD isolated from plasma, demonstrating an apocrine-specific ApoD [10, 14]. Despite the important function of ApoD in the human AO, the relationship between its expression and the strength of the odor is largely unknown.

In this study, we compared the expression of ApoD between the normal and osmidrosis subjects, and exploring the potential regulating mechanisms.

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Materials and methods

Subject classification

This study included 78 AO patients who underwent surgical treatment in the General Hospital of Jinan Military Command between September 2012 and September 2015. Patients were classified into three groups according to the extent of the osmidrosis. Briefly, the osmidrosis patients were asked to stay in a room (with the temperature of 28°C and door closed) and keep the arm naked and exposed. The same examiner was asked to smell the unpleasant odor from different testers sequentially from 5, 3 and 1 meters away. The extents of the osmidrosis were classified into mild, middle, severe degrees corresponding to the unpleasant odor obviously smelled at 1, 3 and 5 meters distances.

Biopsy specimens

Biopsy specimens of the underarm area were obtained from AO patients following local anesthesia with lidocaine hydrochloride containing epinephrine hydrochloride at a dilution of 1:100 000. The tissue was fixed with 4% paraformaldehyde for 72 h, embedded in paraffin and cut into 5- μ m sections.

H&E staining

Routine histological staining with hematoxylin & eosin (H&E) was performed. Briefly, paraffin sections were deparaffinized in xylene (Sigma-Aldrich, Germany), rehydrated with 100%, 90%, 80% alcohols, and rinsed with running water. The sections were then stained with Harris's hematoxylin for 10 min, washed with running water for 5 min, differentiated with 1% acid alcohol, washed with running water for 10 min, counter-stained with 1% eosin (Sigma-Aldrich, Germany) for 5 min. The sections were further dehydrated with 80%, 90%, 100% alcohols, cleared with xylene, mounted in neutral gum, and observed under a microscope (Olympus, Japan) to determine the secretion phases of the apocrine glands. If there were more glands in the active secretion status under three randomly selected visual fields, the subject was dominated as "dominant of active secretion status", otherwise the subject was denoted as "dominant of quiescent secretion status".

Immunohistochemistry (IHC) analyses

The expression level of ApoD in axillary tissues was compared by immunochemical staining. Briefly, the sections were deparaffinized 3 times with xylene, and rehydrated through serial ethanol (100, 90, and 80% ethanol) and water. The sections were heated in antigen retrieval solution in a pressure cooker for 5 min, and incubated in 3% hydrogen peroxide for 8 min to block the endogenous peroxidases. The sections were then blocked with goat serum, incubated with mouse anti-human ApoD antibody (1:60 dilution, Abcam, USA) at 37°C for 3 h. The sections were rinsed and incubated with goat anti-mouse secondary antibody (Zhongshan Biotech., Beijing, China) at room temperature for 2 h, followed by DAB color development (BioGenex, CA, USA), for 6 min. The sections were further counterstained with hematoxylin, dehydrated through serial alcohols, cleared to xylene and cover slipped with mounting resin. The sections were examined for the extent and intensity of staining using a light microscope by two independent observers in a blinded manner. Cytoplasmic staining in yellow or brown was identified as positive staining. The average optical density and integrated optical density (IOD) of five randomly selected visual fields using the Image-Pro Plus 5.0 software.

Quantitative reverse transcription PCR (qRT-PCR)

The expression of ApoD mRNA was detected by qRT-PCR. Briefly, the intact skin from was rinsed with D-Hank buffer, and subcutaneous fat was removed. The skin was minced (1 mm³) with sharp scissors in a culture plate (60 mm in diameter). The pieces were then incubated with type II collagenase (3 mL) at 37°C in a humidified atmosphere of 5% CO₂ and 95% air. On the following day, the gland coils were removed and harvested for RNA isolation. Tissues were rinsed twice in cold sterile PBS. Total RNA was extracted by Trizol (Invitrogen) following the manufacturer's instruction. RNA concentration was determined using a spectrometer under a wavelength of 260 nm. Total RNA was reverse transcribed into cDNA using reverse transcription kit (Promega) according to the manufacturer's instructions. qRT-PCR was performed using an AB 7500 Real-Time PCR System

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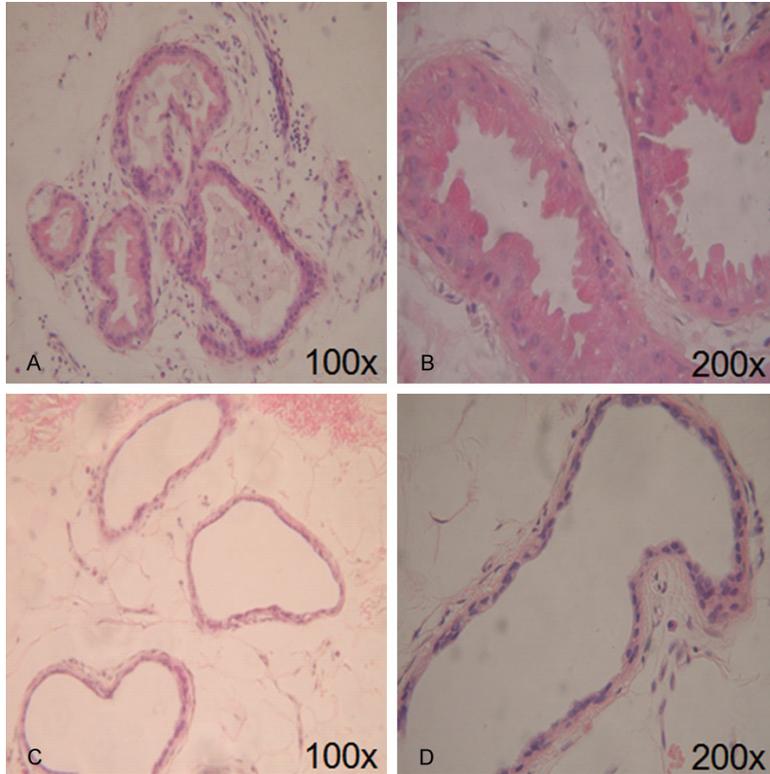


Figure 1. H&E staining comparing the apocrine sweat gland in AO (A, 100× and B, 200×) and control group (C, 100× and D, 200×).

Table 1. Association of AO with the secretion status and ApoD expression of apocrine sweat glands

Parameters	Mild	Middle	Severe	P value
Gender				
Female	7	11	11	P = 0.024
Male	5	13	31	$\chi^2 = 5.094$
Apocrine sweat glands				
Dominant of active secretion status	2	18	39	P < 0.0001
Dominant of quiescent secretion status	10	6	3	$\chi^2 = 25.83$
ApoD mRNA expression				
Low	8	2	2	P < 0.05
Middle	3	15	5	$\chi^2 = 12.06$
High	1	7	35	

(Applied Biosystems, Foster City, CA). Each well contained 12.5 μ l of SYBR Green PCR Master Mix (Takara, Dalian, China), 50 nM sense and antisense primers, 2 μ l of template, and nuclease-free water. Thermal denaturation (melt curve analysis) was used to confirm specific product formation. ApoD mRNA levels were normalized to that of GAPDH. All the relative expression levels from different samples were compared with the sample expressing the

lowest ApoD. The primers for ApoD and GAPDH are listed below: ApoD: F, 5'-TAAACAT-CAGAGACCTGAAG-3', R, 5'-A-GAATCAGCCGATTTGAGAT-3'; GAPDH: F, 5'-AGGCTGTGGG-CAAGGTCATC-3', R, 5'-ACCA-CTGACACGTTGGCAGT-3'. Each sample was run in triplicate. GAPDH was used as an internal control and the relative expression of ApoD was calculated by $2^{-\Delta\Delta CT}$ method. The lowest expression level of all patients was set as control (expression level = 1). The relative ApoD mRNA level was classified into three grades: 1-2 as low expression, 2-4 as medium expression, and higher than 4 as high expression.

Statistical analysis

All measurement data were expressed as mean \pm standard deviation and analyzed using SPSS 16.0 (SPSS Inc, Chicago, IL, USA). Difference between groups was compared by *t* tests. Difference among ratio was compared by chi-square tests. *P* values smaller than 0.05 are considered statistically significant.

Results

The secretion status of the apocrine sweat glands was determined by histological analyses (H&E staining) of axillary tissues. As shown in **Figure 1**, although the apocrine sweat glands were observed in AO and control groups, the area of apocrine sweat glands in the former group was obviously larger compared with the latter. Moreover, the apocrine sweat glands in AO group were active with cells projecting towards the lumen, forming thick walls and small chambers (**Figure 1A** and **1B**). In contrast, the apocrine sweat glands in controls were in quiescent secretion status. The lumens had much thinner wall and larger diameter (**Figure 1C** and

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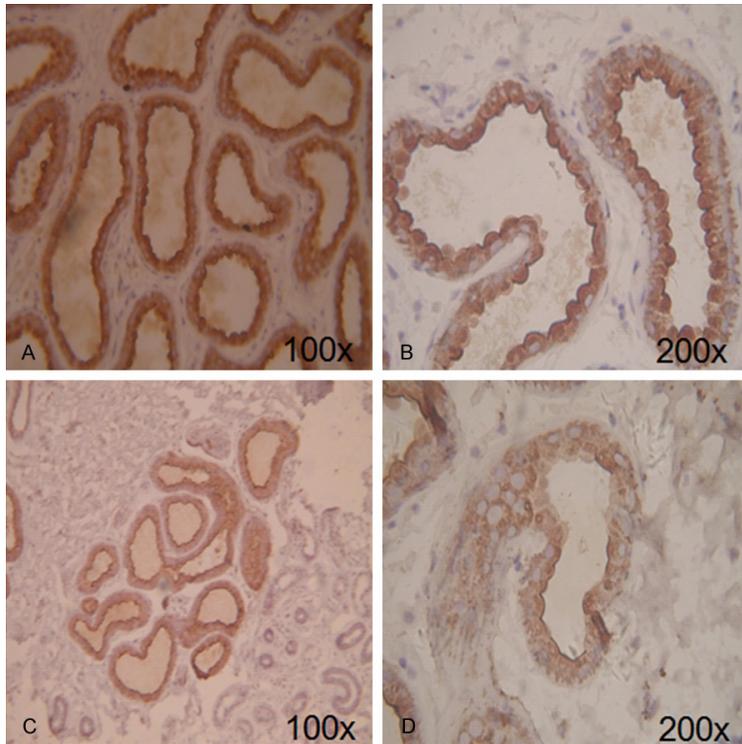


Figure 2. IHC analyses comparing the location and expression of ApoD protein in AO (A, 100× and B, 200×) and control group (C, 100× and D, 200×).

Table 2. IHC analyses comparing the expression of ApoD protein in the apocrine sweat glands in AO and control groups (mean ± standard deviation)

Group	Number of case	Average optical density	Integrated optical density (IOD)
AO	87	0.25 ± 0.07*	167897.29 ± 17323.91*
Control	16	0.11 ± 0.05	25796.42 ± 2948.19

*, P < 0.05 compared with control group.

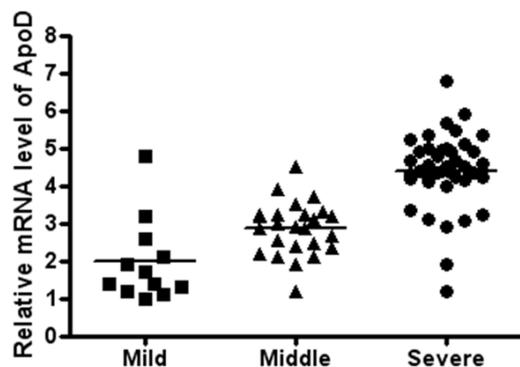


Figure 3. Expression of ApoD mRNA in AO patients. Apocrine sweat glands were isolated from the 78 patients and expression of ApoD mRNA was analyzed by qRT-PCR. GAPDH was used as an internal control and the relative expression of ApoD was calculated

by $2^{-\Delta\Delta CT}$ method. The lowest expression level of ApoD among all patients was set as 1.

1D). Among the 78 patients, there were 59 (75.6%) subjects had their apocrine sweat glands in active secretion status, including 12 mild, 24 middle, and 42 severe patients. The ratio was much higher in the severe patients (39/42, 92.9%), suggesting a strong correlation of the secretion status with the AO. Of the 78 AO patients, 29 were females and 49 were males. Moreover, the proportion of male patients in the severe subgroup was significantly higher compared with that of females ($\chi^2 = 5.094$, $P = 0.024$), indicating a higher incidence of severe AO in male patients (**Table 1**).

Further, the expression of ApoD protein was compared by IHC. A large amount of brown pigimentary granules were observed in the cytoplasm of the apocrine sweat gland cells in AO group (**Figure 2A** and **2B**), whereas only a few positively stained cells were detected in control group (**Figure 2C** and **2D**). The average optical density and IOD in AO group was significantly higher compared with control group ($P < 0.05$, **Table 2**), suggesting a significantly higher ApoD expression in AO group.

We compared the expression of ApoD at mRNA level among these 78 patients by qRT-PCR analyses. The highest level was nearly 6 times higher than the lowest level. The mean ApoD mRNA expression in severe subgroup was significantly higher than the other 2 subgroups ($P < 0.05$, **Figure 3**). Moreover, the proportion of high mRNA expression in severe subgroup was also significantly higher compared with the other subgroups ($\chi^2 = 12.06$, $P < 0.05$, **Table 1**). These results suggested a higher expression of ApoD in severe patients, and thus a correlation between ApoD expression and the severity of AO.

Discussion

In the present study, we demonstrated a strong association between the apocrine gland secretion status, ApoD expression and the severity of AO. Our results suggest that the active secretion of the apocrine glands is one of the important characteristics of AO, which is much prevalent in the severe AO. This finding further confirms that the secretions from the gland should be the origin of the odor.

As to the relationship between gender and AO, there were more male patients in this study, especially the severe patients. Due to the scale of the sample, it is still cautious to deduce that male has a higher incidence, especially when the bias of the mild patient inclusion was considered and the accepted idea that AO is a genetic disorder. However, the prevalence of male patient in the severe group suggests that male patient inclines to display severe manifestation, further pointing to the idea that the microenvironment is also involved in the disease, especially the sex hormones.

In fact, our study revealed that ApoD expression was significantly higher in the severe group. This increase might be explained by both the intrinsic and external stimuli. Genetic clarification of the SNP of ApoD might give an explanation of the increase. Besides, it has been found that ApoD expression is elevated in several cancers, in which it is possibly regulated by sex hormones [15-21]. Furthermore, androgen receptor was found to be abundantly expressed and activated in the apocrine glands [10, 12, 22-26]. It is thus reasonable to deduce that ApoD expression is regulated by the androgen in the apocrine glands, which might explain the higher incidence of male patients falling into the severe group. Further studies unraveling the possibility of AR dependent upregulation of ApoD is now undergoing.

In summary, our study demonstrated a strong association between the active apocrine gland secretion status, higher ApoD expression and the severe extent of AO.

Acknowledgements

This work was supported by the Natural Science Foundation for Training Program of Shandong Province of China (Grant No.: ZR2014HP032),

and the Foundation for President Youth Program of the General Hospital of Jinan Military Command (Grant No.: 2014QNO4).

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Zhen Zhao, Department of Otolaryngology Head and Neck Surgery, 303 Hospital of PLA, 52 Zhiwu Road, Qingxiu District, Nanning 530021, Guangxi, China. Tel: +86-187-1976683; Fax: +86-21-31162012; E-mail: chhzhzh303@163.com

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