Title:

Association between the *rs2590498* polymorphism of Odorant Binding Protein (OBPIIa) gene and olfactory performance in healthy subjects.

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Abstract

Olfactory sensitivity varies by several orders of magnitude among healthy individuals, who may exhibit a reduced sensitivity (hyposmia), a high sensitivity (hyperosmia), or an olfactory blindness (anosmia). Environmental and genetic factors seem to account for this variability. Most of odorant molecules are hydrophobic and it has been suggested that odorants are transported to the olfactory receptors by means of odorant binding proteins (OBPs). Aim of this study was to evaluate the presence of a relationship between the olfactory performance of healthy subjects and the polymorphism in the odor binding-protein (OBPIIa) gene, the only OBP found in the olfactory epithelium of humans. Using the "Sniffin' Sticks" Extended Test we assessed the olfactory performance in 69 subjects, who were genotyped for the *rs2590498* polymorphism of the *OBPIIa* gene, whose major allele A has been associated with a higher retronasal perception as compared to the minor allele G. We found that subjects homozygous for the A-allele exhibited threshold scores higher than subjects homozous for the G-allele or heterozygous. In addition, subjects classified as normosmic and hyposmic differed on the basis of genotype distribution and allelic frequencies. In fact, a normosmic condition was associated with genotype AA and allele A and a hyposmic condition was associated with genotype GG and allele G. In conclusion, our results show that a relationship exists between the physiological variations of olfactory performance and the *OBPIIa* gene polymorphism.

Keywords physiological variations of olfactory performance, OBPIIa odorant binding proteins, olfactory threshold, Sniffin' Sticks test, anosmia, olfaction.

1. Introduction

Smell and taste play a primary role in the survival of many species of invertebrates and vertebrates, including humans [1-8]. Olfactory and gustatory information allows animals to locate and choose food, identify predators, select a partner for mating, mother-infant recognition and to avoid potentially harmful situations [9-17].

Most of living organisms, from invertebrates to mammals, have the ability to smell and recognize a great variety of odorants [9, 18-22]. In humans, the perception of odors is conditioned by several factors including personal experiences or environmental variables [23]. This issue is even more complex if we consider that human olfactory perception, especially in terms of intensity and pleasantness, differs enormously among individuals [24-29]. The olfactory sensitivity can vary by several orders of magnitude; in fact, individuals may exhibit a reduced sensitivity (hyposmia), a high sensitivity (hyperosmia), a total or specific olfactory blindness (general or specific anosmia) [19, 27, 30-33]. This variability appears to be due to environmental and genetic factors [6, 9, 19, 28, 34-36].

Olfactory perception begins with the activation of the olfactory receptors (ORs) by the odorant molecules. ORs are localized in the ciliated end of the olfactory sensory neurons (OSNs) [37]. OSNs are cells housed in the olfactory epithelium, which is covered by a thin layer of dense mucus, rich in glycoproteins that retain water and protect against foreign agents [6, 38]. This region surrounding the OSNs is called "perireceptor space" and the chemical interactions between odors and soluble macromolecules contained in this space are called "perireceptor events" [38-39]. These events include: variation in the composition and thickness of the mucus, presence of enzymes that modify the chemical structure and/or concentration of odorants, and the presence of odorant binding proteins (OBPs) that can alter the accessibility of odorants to sensory cells [38, 40-44]. The majority of odorant molecules is hydrophobic. Still they need to cross the mucus barrier to activate the ORs and thus initiate the olfactory transduction process. Some authors suggested that odorants are captured and transported through the mucus layer by OBPs [18, 38, 45-47] (, which are highly

expressed in the olfactory cleft [48]. Contrary to what has been found in other animal species where different subtypes of OBPs are simultaneously present in the mucus [49-53], the only OBP found in the mucus surrounding the olfactory epithelium in humans is the OBPIIa [6, 54]. The polymorphism (*rs2590498*) of the gene coding for the human OBPIIa has been associated with variations of retronasal perception: the major allele A, compared to the minor allele G, shows a higher sensitivity [55]. Furthermore, little is known about the relationship between the individual olfactory sensitivity and the polymorphism of OBPs, unlike the different polymorphisms of ORs, which represent the molecular basis for inter-individual variations in olfactory perception to specific odorants [19, 26-27, 56].

On the basis of these lines of evidence, the aim of the current study was to investigate the association between the *rs2590498* polymorphism of *OBPIIa* locus and overall olfactory performance, odor threshold, odor discrimination and odor identification.

2. Materials and Methods

2.1 Subjects

Sixty-nine caucasian healthy non-smoking volunteers (53 females and 16 males), aged 19-55 years (31.7 ± 1.29 years), recruited in Cagliari (Sardinia, Italy), took part in the study. All subjects were free of perfumes and fasted for at least 2 hours prior to testing. Besides, they were informed of all the experimental procedures and signed a written informed consent. The study was conducted in accordance with the Declaration of Helsinki of 1975 (revised in 1983) for research involving humans and was approved by the Ethical Committee of the University Hospital of Cagliari.

2.2 Olfactory sensitivity screening

The orthonasal olfactory function of each subject was evaluated by means of the Sniffin' Sticks Extended Test (SSET; Burghart Instruments, Wedel, Germany), which is based on odor-containing felt-tip pens [57]. The SSET comprises three subtests: Threshold, Discrimination and Identification

test (TDI), which are presented in this order. For odor presentation the pen's cap was removed and the pen's tip was placed approximately 2 cm in front of both nostrils for about 3 s. In addition, during the olfactory threshold and discrimination tests, the subjects were blindfolded to prevent the visual identification of the pens containing the odorant in question [58]. The olfactory threshold was assessed using a single-scale, triple-forced choice paradigm. The experimenter had 16 triplets of pens (16 increasing concentrations of odorant). In each triplet two pens contained the solvent and the third pen the odorant (n-butanol). The participant's task was to identify the pen containing the odorant. Triplets were presented at 20 s intervals. Reversal of the staircase was validated if the odorant was correctly identified by the participant two times in a row. The test ended when 7 reversals were reached. The olfactory threshold is defined as the mean of the dilution steps at the last 4 reversals. The score assigned to each participant was between 1 and 16. Odor discrimination was also assessed by 16 triplets of pens: in this case two pens were filled with the same odor and the third with a different one (target pen). The objective was to identify, for each triplet, the pen containing the different odor. The triplets were presented every 20-30 sec and the interval between the pens within the same triplet was about 3 sec. The score obtained corresponds to the number of correct answers from 0 to 16. The olfactory identification was assigned by means of 16 pens containing common odors. The participants had to identify, for each pen, the odor smelled using a four-alternative forced choice from flash cards that had both the picture and name of the object. The score corresponds to the number of correct identifications.

The total score obtained in the three subtests (TDI) or that of each subtest (threshold subtest, T; Discrimination subtest, D; Identification subtest, I) were used to classify the participants as normosmic, hyposmic or functionally anosmic (further termed "anosmic") for the overall olfactory performance or their olfactory threshold, discrimination and identification performance, respectively. The reference values used for classifying subjects into the three olfactory function groups (normosmic, hyposmic or anosmic) took into account sex and age according to previous investigations [58-59].

2.3 Genetic analysis

The QIAamp® DNA Mini Kit (QIAGEN S.r.l., Milan, Italy) was used to extract DNA from saliva samples, in accordance with the manufacturer's instructions. Subjects were genotyped for the *rs2590498* (A/G) polymorphism of *OBPIIa* gene using a custom TaqMan® SNP Genotyping Assay (Applied Biosystems by Life-Technologies Italia, Europe BV) according to our previous investigations [55, 60]. Briefly: forward PCR Primer GCCAGGCAGGGACAGA and Reverse PCR primer CTACACCTGAGACCCCACAAG were used; Two TaqMan probes were designed according to the *OBPIIa* gene (bold and underlined), probe/reporter 1: VIC-TCGGTGAC**A**TGAACC and probe/reporter 2: FAM–TCGGTGAC**G**TGAACC. After PCRs, the fluorescence of plates was read by the sequence detector system at 60 °C for 1 min and the results analyzed by allelic discrimination of the sequence detector software (Applied Biosystems). Positive and negative controls and replicates were included in the reactions.

2.4 Statistical analysis

Stepwise, multiple linear regression was used to determine the relative contribution of T, D and I scores as predictor variables on TDI score. The relative contribution of each significant variable and semipartial correlations (sr) for each variable are reported in Table 2.

An analysis of variance for repeated measures (ANOVA) was used to analyze differences of the T, D and I scores (within subject factor) in relation to *OBPIIa* genotype groups (between subject factor). Data were checked for the assumptions of homogeneity of variance, sphericity and normality. When the sphericity assumption was violated, a Greenhouse-Geisser correction or Huynh-Feldt correction was applied to modify the degrees of freedom. Post-hoc comparisons were conducted with the Fisher's least significant difference (LSD) test, unless the assumption of homogeneity of variance was violated, in which case Duncan's test was used. Statistical analyses

were performed using STATISTICA for WINDOWS (version 7.0; StatSoft Inc, Tulsa, OK, USA). P values < 0.05 were considered to be significant.

Differences on genotype distribution and allele frequencies at the *OBPIIa* locus between subjects classified as normosmic or hyposmic for the TDI olfactory status, and singularly for the T, D and I status, were analyzed using Fisher's method (Genepop software version 4.2; http://genepop.curtin.edu.au/genepop_op3.html) [61].

3. Results

Distribution of subjects classified as normosmic and hyposmic based on their overall olfactory status and their single T, D or I status is shown in Table 1. In detail, based on TDI scores 45 subjects (65.2%) were classified as normosmic and 24 (34.8%) as hyposmic. Instead, based on the score obtained from each subtest, 28 subjects were classified as normosmic (40.6%) and 41 hyposmic (59.4%) by means of T score, 61 (88.4%) subjects were classified as normosmic and 8 as hyposmic (11.6%) by means of both D and I scores. None were classified as anosmic.

Multiple linear regression showed the relative contribution of each subtest score on the TDI score (Table 2). In particular, the score of T, D and I subtests contributed 50.3%, 31.0% and 17.0%, respectively to the model.

Molecular analyses for the *rs2590498* (A/G) polymorphism of the *OBPIIa* gene allowed us to identify the genotype of the 69 subjects: 20 were homozygous AA, 18 were heterozygous and 31 were homozygous GG. The relationship between the *rs2590498* (A/G) polymorphism of *OBPIIa* locus and olfactory threshold, discrimination or identification performance, is shown in Fig. 1. The repeated-measures ANOVA revealed a significant interaction between T, D and I scores and the *OBPIIa* genotypes ($F_{3.8,126.9} = 5.01$, $p = 0.0009$). Post-hoc comparisons showed that the subjects that were homozygous for the A-allele exhibited T scores higher than subjects who were homozygous for the G-allele ($p = 0.00003$; Fisher's LSD test) or heterozygous ($p < 0.00001$; Fisher's LSD test).

Genotype distributions and allele frequencies for the *rs2590498* (A/G) polymorphism of *OBPIIa* gene according to TDI score are shown in Table 3. Subjects classified as normosmic and hyposmic differed on the basis of genotype distribution (χ^2 = 6.47, p = 0.039; Fisher's method) and allelic frequencies (χ^2 = 9.23, p = 0.01; Fisher's method). In addition, Fisher's method showed that significant differences based on the genotype distribution and allele frequencies of the *OBPIIa* locus also exist between subjects classified as normosmic and hyposmic by means on the T and I subtest (T: χ^2 = 6.168; p = 0.046 and χ^2 = 8.811; p = 0.012; I: χ^2 = 6.199; p = 0.045 and χ^2 = 8.856; p = 0.012; Fisher's method), but not when subjects were classified by D performance (χ^2 = 4.134; P = 0.126 and χ^2 = 5.752; p = 0.056; Fisher's method) (Table 3).

4. Discussion

Previous reports showed a great variability in the olfactory performance in healthy humans [33]. Consistently, in subjects who participated in this study we found variations in olfactory performance: 35% of subjects were hyposmic and 65% were normosmic, when they were tested with the complete SSET. Instead, the percentage of hyposmic and normosmic subjects determined by each subtest was: 59 and 41% when T was considered and 88 and 12% in the case of D or I, thus suggesting that healthy subjects exhibit a great variability in olfactory threshold.

The main goal of the present work was to study the olfactory performance of healthy subjects in relation to the *rs2590498* (A/G) polymorphism of the *OBPIIa* locus, with the aim of understanding whether the physiological variability of olfactory sensitivity can be considered, at least in part, as the phenotypic display resulting from the allelic diversity of the gene coding for the human common odorant-binding protein *OBPIIa*. It is known that OBPs act as carriers and facilitate the odor-receptor binding by transporting the odor molecules to the receptor site [18]. Our results highlight a direct relationship between the *OBPIIa* polymorphism and the olfactory performance of healthy subjects. Gene polymorphisms have been already described as a mechanism by which individuals exhibit functional variation within a physiological range [28, 62-63]. In our sample we

found a different genotype distribution and allele frequencies between subjects classified as normosmic or hyposmic, which display a higher orthonasal olfactory performance associated with genotype AA and allele A and a hyposmic condition associated with genotype GG and allele G. Moreover, by means of a comparative analysis we associated the highest olfactory performance determined by T score with genotype AA and the lowest threshold performance with genotype AG or GG. In fact, we found that subjects who were homozygous for the A-allele obtained scores in T test that were significantly higher than those of the other two genotypes, but not in the D and I tests. These results are in agreement with previous studies showing that the major allele A is associated with a higher retronasal perception as compared to the minor allele G [55]. In fact, our findings indicate that the presence of at least a G allele is sufficient to decrease in healthy subjects the olfactory performance determined with the T test, which was the main determinant of the overall performance. The AA homozygous genotype seems to be most important for olfactory threshold ability, which represents the minimum concentration at which an odor is perceived [64-65]. Because the odorant-OR binding initiates the transduction process and triggers the peripheral sensory activation giving rise to the ultimate perception of odors [66-67], subjects having the testing variant of OBPIIa would be able to perceive the odor also when it is present at very low concentrations. In contrast, no effect of this polymorphism was found on the ability to discriminate or identify odors, as already observed in a previous study in healthy subjects [68]. This is in agreement with the notion that the abilities to discriminate and identify odorants are related to higher olfactory functions which are more strongly correlated with cognitive factors as compared to odor thresholds [33, 65].

In conclusion, the results of this work highlight for the first time that the physiological variations of overall olfactory performance exhibited by healthy subjects can be, at least in part, of genetic origin, and identify in the *rs2590498* (A/G) polymorphism of the *OBPIIa* a candidate for the individual changes of olfactory sensitivity. Therefore, olfactory variability could be governed in part by peri-

receptor events, such as the polymorphism of the *OBPIIa* proposed by these results, but also by mechanisms and processes linked to olfactory cognition at the level of the central nervous system.

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Conflict of interest

There are no financial and personal relationships with other people or organizations that may lead to a conflict of interest.

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Figure legends

Figure 1. Effect of the *OBPIIa* genotypes on the T, D and I score.

Mean values ± SE of Threshold (T), Discrimination (D) and Identification (I) score according to genotypes of the OBPIIa locus. n = 69 (31 GG, 18 AG, 20 AA). Different letters indicate significant differences ($p < 0.0001$; Fisher's LSD test).

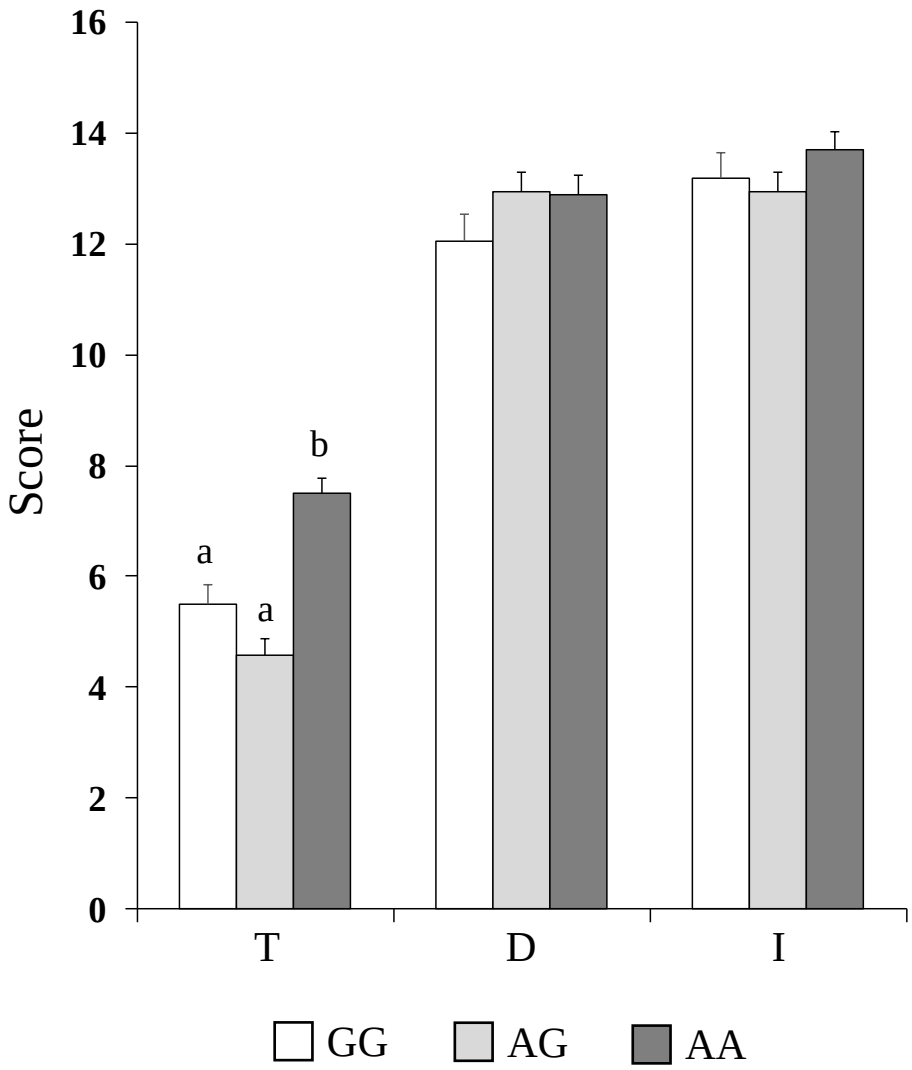

Figure 1

Table 1. Distribution of the subjects (n = 69) classified as normosmic or hyposmic based on their overall olfactory status (TDI), Threshold (T), Discrimination (D) or Identification (I).

	Olfactory status	
	Normosmic n (%)	Hyposmic n (%)
TDI	45 (65.22)	24 (34.78)
Threshold	28 (40.58)	41 (59.42)
Discrimination	61 (88.41)	8 (11.59)
Identification	61 (88.41)	8 (11.59)

Table 2. Stepwise forward multiple regression models for TDI score.

TDI score	Variable	Overall model		Parameter estimate		Each step
		(adj R^2)	(p)	(sr)	(p)	(R^2)
	T	0.9832	<0.00001	0.6682	<0.00001	0.5028
	D			0.4404	<0.00001	0.8132
	I			0.4065	<0.00001	0.9832

Indipendent variables included: Threshold (T), Discrimination (D) and Identification (I) score. Adj = adjusted; sr = semipartial correlation.

Table 3. Genotype distribution and allele frequencies of the *rs2590498* polymorphism of the *OBPIIa* gene (A/G) in the subjects classified as normosmic or hyposmic on the basis of the TDI score obtained.

TDI	Normosmic n (%)	Hyposmic n (%)	P-value[a]
Genotype			0.0394
AA	18 (40.00)	2 (8.33)	
AG	9 (20.00)	9 (37.50)	
GG	18 (40.00)	13 (54.17)	
Allele			0.0099
A	45 (50.00)	13 (27.08)	
G	45 (50.00)	35 (72.92)	

[a] P-value derived from Fischer's method. Genotype AA: n = 20; Genotype AG: n = 18; Genotype GG: n = 31.

T	Normosmic n (%)	Hyposmic n (%)	P-value[a]
Genotype			0.0458
AA	15 (53.57)	5 (12.20)	
AG	1 (3.57)	17 (41.46)	
GG	12 (42.86)	19 (46.34)	
Allele			0.0122
A	31 (55.36)	27 (32.93)	
G	25 (44.64)	55 (67.07)	

[a] P-value derived from Fischer's method. Genotype AA: n = 20; Genotype AG: n = 18; Genotype GG: n = 31.

D	Normosmic n (%)	Hyposmic n (%)	P-value[a]
Genotype			0.1264
AA	19 (31.15)	1 (12.50)	
AG	17 (27.87)	1 (12.50)	
GG	25 (40.98)	6 (75.00)	
Allele			0.0564
A	55 (45.08)	3 (18.75)	
G	67 (54.92)	13 (81.25)	

[a] P-value derived from Fischer's method. Genotype AA: n = 20; Genotype AG: n = 18; Genotype GG: n = 31.

I	Normosmic n (%)	Hyposmic n (%)	P-value[a]
Genotype			0.0451
AA	20 (32.79)	0 (/)	
AG	16 (26.23)	2 (25.00)	
GG	25 (40.98)	6 (75.00)	
Allele			0.0119
A	56 (45.91)	2 (12.50)	
G	66 (54.09)	14 (87.50)	

[a] P-value derived from Fischer's method. Genotype AA: n = 20; Genotype AG: n = 18; Genotype GG: n = 31.