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Association of Smoking Behavior with an Odorant Receptor Allele Telomeric to the Human Major Histocompatibility Complex

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Smoking behavior has been associated in two independent European cohorts with the most common Caucasian human leukocyte antigen (HLA) haplotype (A1-B8-DR3). We aimed to test whether polymorphic members of the two odorant receptor (OR) clusters within the extended HLA complex might be responsible for the observed association, by genotyping a cohort of Hungarian women in which the mentioned association had been found. One hundred and eighty HLA haplotypes from Centre d'Etude du Polymorphisme Humain families were analyzed *in silico* to identify single-nucleotide polymorphisms (SNPs) within OR genes that are in linkage disequilibrium with the A1-B8-DR3 haplotype, as well as with two other haplotypes indirectly linked to smoking behavior. A nonsynonymous SNP within the *OR12D3* gene (rs3749971^{T}) was found to be linked to the A1-B8-DR3 haplotype. This polymorphism leads to a ^{97}Thr → Ile exchange that affects a putative ligand binding region of the OR12D3 protein. Smoking was found to be associated in the Hungarian cohort with the rs3749971^{T} allele ($p = 1.05 \times 10^{-2}$), with higher significance than with A1-B8-DR3 ($p = 2.38 \times 10^{-2}$). Our results link smoking to a distinct OR allele, and demonstrate that the rs3749971^{T} polymorphism is associated with the HLA haplotype–dependent differential recognition of cigarette smoke components, at least among Caucasian women.

Introduction

There were nearly 1.3 billion smokers worldwide in the year 2003, and this number is expected to rise to 1.7 billion (~1.2 billion males and 500 million females) by 2025, with the number of female smokers contributing most to the increase (American Cancer Society, 2003). In nearly all investigated regions of the world, the ratio of female to male smokers among young people was found to be higher than the ratio among adults, suggesting a global trend for an increase in smoking habits among female adolescents and young women (Global Youth Tobacco Survey Collaborating Group, 2003). Smoking is associated with many serious health problems, including cancer of various organs, coronary artery disease, as well as several autoimmune disorders (Hegediüs *et al.*, 2004; Klareskog *et al.*, 2006; Warren *et al.*, 2006; American Cancer Society, 2007; Koch *et al.*, 2007; Hawkes, 2007), and it is thus considered a leading cause of death and disability worldwide. Although there is general agreement that nicotine is the core addictive component of cigarette smoke (Jarvis, 2004), there are hundreds of further substances that may influence the initiation and continuation of tobacco abuse (Baker *et al.*, 2004), independent of nicotine (Franklin *et al.*, 2007). Smoking is also modulated by genetic factors, as demonstrated by epidemiological and twin studies (Sullivan and Kendler, 1999; Li *et al.*, 2003). In support, a haplotype of the major histocompatibility (human leukocyte antigen, HLA) complex was found to be associated with smoking behavior, stronger in women (odds ratio: 13.6) than in men (odds ratio: 2.79) (Füst *et al.*, 2004). This haplotype, –HLA-A1-B8-DR3–, the most common among Caucasians (Alper *et al.*, 2006), is also associated with autoimmune disorders, of which some are clearly connected with tobacco abuse, such as Graves' ophthalmopathy (Weetman, 2000; Hunt *et al.*, 2001; Hegediüs *et al.*, 2004; Holm *et al.*, 2005).

At least two further HLA haplotypes have also been linked to autoimmune diseases that are triggered or heavily influenced by tobacco smoking. The HLA-A3-B7-DR15 haplotype

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is overrepresented in individuals with multiple sclerosis (Dyment *et al.*, 2004; Herrera *et al.*, 2006), while HLA-DR "Shared Epitope" (SE) haplotypes are overrepresented in individuals who smoke, possess antibodies against citrullinated proteins, and suffer from rheumatoid arthritis (Klareskog *et al.*, 2006; Linn-Rasker *et al.*, 2006). SE haplotypes are characterized by the presence of the HLA-DRB1*01, -DRB1*04, or -DRB1*10 alleles.

The exceptionally strong linkage disequilibrium (LD) that is typical for certain haplotypes of the xHLA encompasses a chromosomal segment with a length of about 7 Mb between the gene *HFE* and loci within the HLA class II region (Horton *et al.*, 2004). In case of the A1-B8-DR3 haplotype, LD is extreme over its entire length (Alper *et al.*, 2006), suggesting that an allele of any genes within the region of LD could in principle predispose to smoking. Therefore, polymorphic members of the two odorant receptor (OR) gene clusters within the telomeric xHLA (Ehlers *et al.*, 2000; Younger *et al.*, 2001; Ziegler and Uchańska-Ziegler, 2006) must be considered as plausible candidate genes.

The Centre d'Etude du Polymorphisme Humain (CEPH) panel of families (Miretti *et al.*, 2005) offers unique opportunities for genetic association studies comprising the HLA region. The samples analyzed here comprise 180 founder chromosome 6 that have already been analyzed with regard to their single-nucleotide polymorphism (SNP) alleles and tagSNPs (representative SNPs for a genomic region exhibiting high LD) (Miretti *et al.*, 2005). Recently, the analysis has been extended to both OR gene clusters at the telomeric section of the xHLA, permitting the correlation with alleles within the HLA class I, II, and III regions (de Bakker *et al.*, 2006).

The present study was conducted in two steps: (i) *in silico*, we aimed to identify SNP alleles within the two HLA-linked OR gene clusters that are characteristic for the HLA haplotypes mentioned before; and (ii) *in vitro* we wanted to test whether their possible association with tobacco abuse is stronger or weaker than the one observed with A1-B8-DR3, by genotyping a subsample of the cohort in which an association between smoking and loci at the HLA class III region had been found before.

Materials and Methods

In silico *tagSNP selection and assessment of HLA haplotype–dependent OR SNP alleles*

All *in silico* analyses were based on xHLA high-density SNP genotyping data from 180 founder chromosome 6 from the CEPH collection (Miretti *et al.*, 2005). Details on marker selection, CEPH subjects, and on their genotypings are given elsewhere (Miretti *et al.*, 2005; de Bakker *et al.*, 2006). A total of 1170 SNPs spanning the region between *OR2B2* and *MOG* (Fig. 1) were initially considered and analyzed with regard to their allelic diversity (supplemental Fig. S1, available online at www.liebertpub.com). A list of all 1170 SNPs with their genomic coordinates is given in the supplemental Table S1 (available online at www.liebertpub.com). From this set of markers, we chose 110 tagSNPs capturing the haplotypic information from the whole genomic region. SNP tagging was performed using a pairwise tagging algorithm (de Bakker *et al.*, 2006), as implemented in HAPLOVIEW v. 4 (Barrett *et al.*, 2005), and considering a maximum intermarker distance of 650 kb and an LD coefficient (r^2) threshold of $\geq$0.8. Loci with a minor allele frequency of less than 1%, those not conforming to Hardy–Weinberg equilibrium, or not reported by the dbSNP database (http://www.ncbi.nlm.nih.gov/projects/SNP) were excluded.

We tested the 110 tagSNPs characterizing the *OR2B2-MOG* segment for association with three groups of haplotypes (A1-B8-DR3, A3-B7-DR15, and SE), the aim being to determine which tagSNPs were characteristic for each of the three haplotype groups.

Genotyping of SNP rs3749971

The genotyping of the SNP *rs3749971* was performed by real-time PCR in a sample of 32 Hungarian female Caucasians (average age 46.75 years, ranging from 24 to 76 years), which

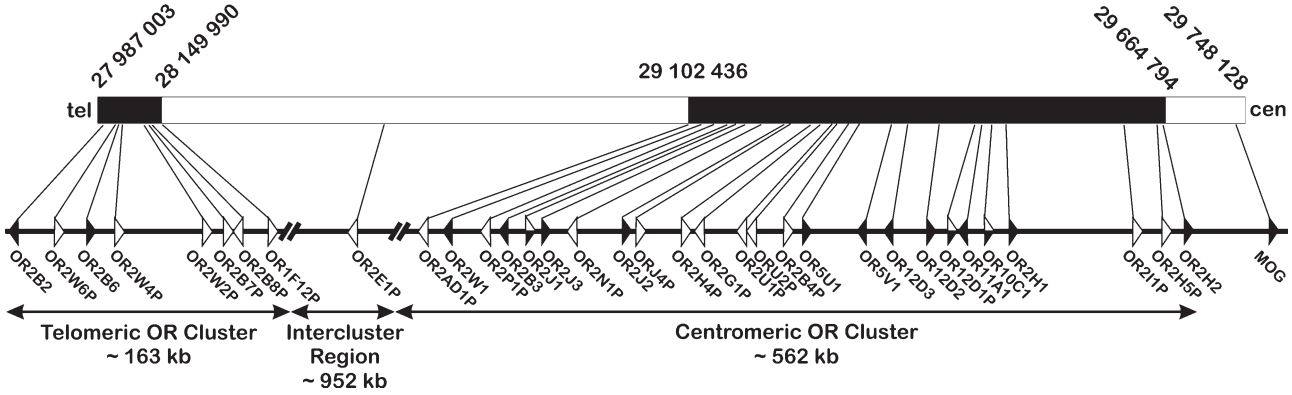

FIG. 1. Map (not to scale) of the region encompassing the two OR clusters on chromosome 6p. Above the figure, the NCBI 36 coordinates (bp) of the chromosomal segments analyzed here are depicted. The upper plot is filled in black for OR clusters and in white for regions outside the OR clusters, while the lower part of the figure shows the OR genes/pseudogenes at their relative chromosomal locations (suppressing most of the intercluster region). Triangles indicate transcriptional orientation, and are filled in black (genes), white (pseudogenes), or in both colors (haplotype-dependent genes/pseudogenes). tel: direction to the telomere; cen: direction to the centromere.

was a subsample of a cohort in which a correlation had previously been found between smoking and loci of the HLA class III region (Füst *et al.*, 2004). Based on LD, women who carried the C4A*Q0 (mono-S) genotype as well as the AGER-429C, HSPA1B-1267G, and TNF-308A alleles were considered carriers of the A1-B8-DR3 haplotype (Füst *et al.*, 2004). Other genotypings of these subjects, details on registration of smoking habits, preparation of genomic DNA, as well as informed consent from the cohort have been provided before (Füst *et al.*, 2004).

The PCR reactions were performed using a Stratagene real-time PCR instrument (Stratagene, Amsterdam, NL) with the following cycle conditions: 1 cycle of 94°C for 1 min and 80 cycles of 94°C for 20 s, 62°C for 20 s, 72°C for 30 s, and 78°C for 10 s. Fluorescence was measured during the 62°C (annealing) step. Twenty-microliter PCR reactions contained 100 ng of human genomic DNA; 1.5 pmol C- or T-specific reverse primers (details below); 3 pmol forward primer (rs3749971-For: AGCGAAGAGGATTGCAGATGGC); 2 μL Genetherm polymerase buffer; 0.7 mM each of dATP, dCTP, dGTP, and dTTP; 2 mM MgCl; and 2 U of GenTherm™ Taq Polymerase. Allele-specific primers were designed as molecular beacons (Jordens *et al.*, 2000) (rs3749971-C: Fam-atacagc CTATATCTTTTCTAGGCTGTA***T***$_{BHQ}$CA**C** and rs3749971-T: Hex-atacagcCTATATCTTTTCTAGGCTGTA***T***$_{BHQ}$CA**T**), labeled either with FAM (6-carboxyfluorescein) or Hex (4,7,2′,4′,5′,7′-hexachloro-6-carboxyfluorescein), and quenched with Black Hole Quencher (BHQ™) attached to a ***T*** natively present within the primer binding site. Seven bases were added to the 5′ end (displayed in lower-case letters) to allow the formation of a 29-bp "hairpin" with the 10-bp complementary region to ensure fluorescence quenching of the unused primers. To assess the reproducibility of this genotyping approach, control DNA of seven individuals (including three CEPH samples) was typed 15 times independently, with 100% reproducibility.

Statistical analyses

Nucleotide diversity (π) was calculated as described by Nei (1987), using the software DNAsp (Rozas and Rozas, 1999). The two-sided Fisher's exact test was employed for all association analyses, with a 1% level of significance. The Bonferroni correction for multiple comparisons was applied for *p*-values of the *in silico* analyses only.

Results

In silico *analyses*

The degree of nucleotide diversity as assessed by 1170 SNPs (elicited in the supplemental Table S1) was found to be highest between the genes *OR12D3* and *OR10C1* (supplemental Fig. S1), including the most polymorphic loci within this cluster (Ehlers *et al.*, 2000). The SNP diversity outside of the OR clusters did not differ substantially from that within the clusters. SNP densities within the telomeric (0.388/kb) and the centromeric OR clusters (0.877/kb) were relatively high when compared with other genomic regions (Zhao *et al.*, 2003).

Within the panel of 180 xHLA haplotypes, 11 A1-B8-DR3, 5 A3-B7-DR15, and 59 HLA-DR SE haplotypes were found. No significant association between any of the tagSNPs and the 59 HLA-DR SE haplotypes was observed, as shown in Figure 2. A similar result was obtained with regard to the A3-B7-DR15 haplotypes, although this could be due to the low number of A3-B7-DR15 haplotypes (five) in the CEPH panel. In contrast, a significant correlation was found for 12 tagSNPs (representing 81 tagged SNPs displayed in supplemental Table S2, available online at www.liebertpub.com) when the 11 A1-B8-DR3 haplotypes were compared with the 169 non–A1-B8-DR3 haplotypes for association with the 110 tagSNPs (Fig. 2), and after setting a Bonferroni cutoff for multiple comparisons. Only one of the 81 captured SNPs is a coding, nonsynonymous SNP (*rs3749971*, tagged by tagSNP #51), within the gene *OR12D3*. In A1-B8-DR3 haplotypes, the respective allele (rs3749971^{T}) is responsible for a Thr→Ile exchange at amino acid position 97 within the OR12D3 protein. The remaining 80 SNPs lead either to synonymous exchanges or are located in intergenic or in intronic regions. Because none of these are, to our knowledge, directly involved in any, so far known, biological process, they were not considered further.

Since the A1-B8-DR3 haplotype was reported in another independent European cohorts to be overrepresented in

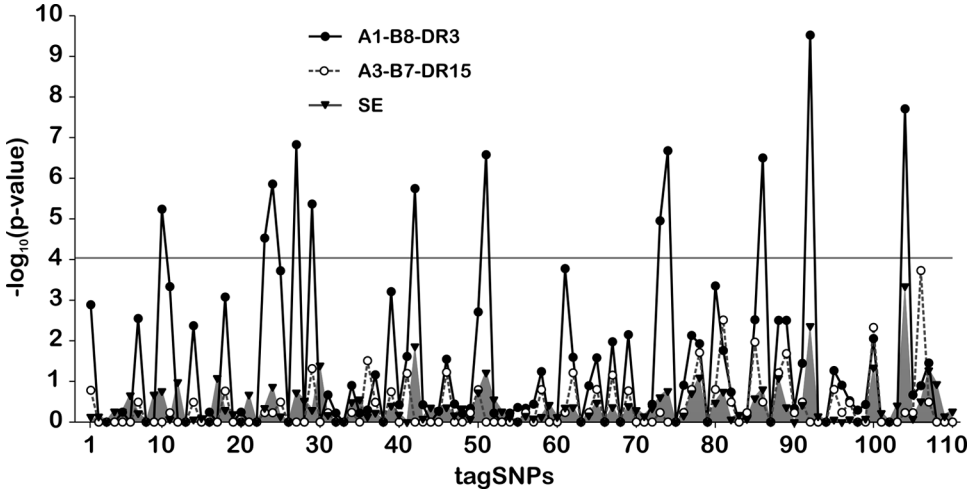

FIG. 2. Allelic association of 110 tagSNPs from the region comprising both HLA-linked OR clusters with A1-B8-DR3, A3-B7-DR15, and SE haplotypes. The marker *rs3749971* is represented by tagSNP #51. The gray horizontal line marks the Bonferroni threshold for 110 tests with a significance level of 1%.

smokers (Icelandic sample, Füst *et al.*, 2004), and is also associated with various autoimmune diseases, of which some correlate with this behavioral trait, as in Graves' disease (Hegediüs *et al.*, 2004; Holm *et al.*, 2005), this data demonstrate that these associations must also extend to the A1-B8-DR3–associated rs3749971^{T} allele.

Cohort genotyping

In order to validate this finding, comparing it to the previously found association, we genotyped *rs3749971* in 32 female individuals from the same Hungarian cohort (Füst *et al.*, 2004). These individuals differ in their smoking behavior and do not suffer from any autoimmune disease (Füst *et al.*, 2004). Here we found that the correlation between smoking and rs3749971^{T} was slightly stronger ($p = 1.05 \times 10^{-2}$, Fig. 3b) than the correlation between this trait and A1-B8-DR3 ($p = 2.38 \times 10^{-2}$, Fig. 3c). Seven and 25 subjects were rs3749971$^{C/T}$ and rs3749971$^{C/C}$ carriers, respectively. A strong association ($p = 5.87 \times 10^{-4}$) between the rs3749971^{T} allele and the A1-B8-DR3 haplotype was observed, as five out of seven of the rs3749971^{T} carriers but only 1/25 of the noncarriers were found to be A1-B8-DR3 positive (Fig. 3d). The group of rs3749971$^{C/C}$ carriers includes both smokers and nonsmokers, as shown in Figure 3a and b.

Discussion

HLA-A1–positive individuals, in contrast to those with HLA-A2 or HLA-A3, have been reported to exhibit a preference for the odor of bergamot (Milinski and Wedekind, 2001). The existence of a relationship between rs3749971^{T} (or OR12D3^{97Ile}) and this predilection for a perfume ingredient is supported by the strong LD between HLA-A1 and rs3749971^{T} that is described here. The fact that many tobacco brands are scented with bergamot oil components (Baker *et al.*, 2004) provides a plausible explanation for the correlation of rs3749971^{T} with smoking, and the identification of OR12-D3^{97Ile} ligands will facilitate the design of volatiles that might be used as antidotes in individuals with a predisposition to tobacco abuse. The exchange of the hydrophilic amino acid threonine by isoleucine, a residue with a hydrophobic side chain, is expected to alter the physicochemical properties of the OR12D3 protein. However, as no X-ray crystallographic studies of ORs have been reported to date, the likely location of ^{97}Ile within the OR12D3 protein can only be inferred from models (Man *et al.*, 2004; Katada *et al.*, 2005; Abaffy *et al.*, 2007; Schmiedeberg *et al.*, 2007) that take the structure of rhodopsin (Palczewski *et al.*, 2000) into account. Such models locate residue 97 at the end of the first extracellular loop or at the beginning of the third transmembrane domain, close to

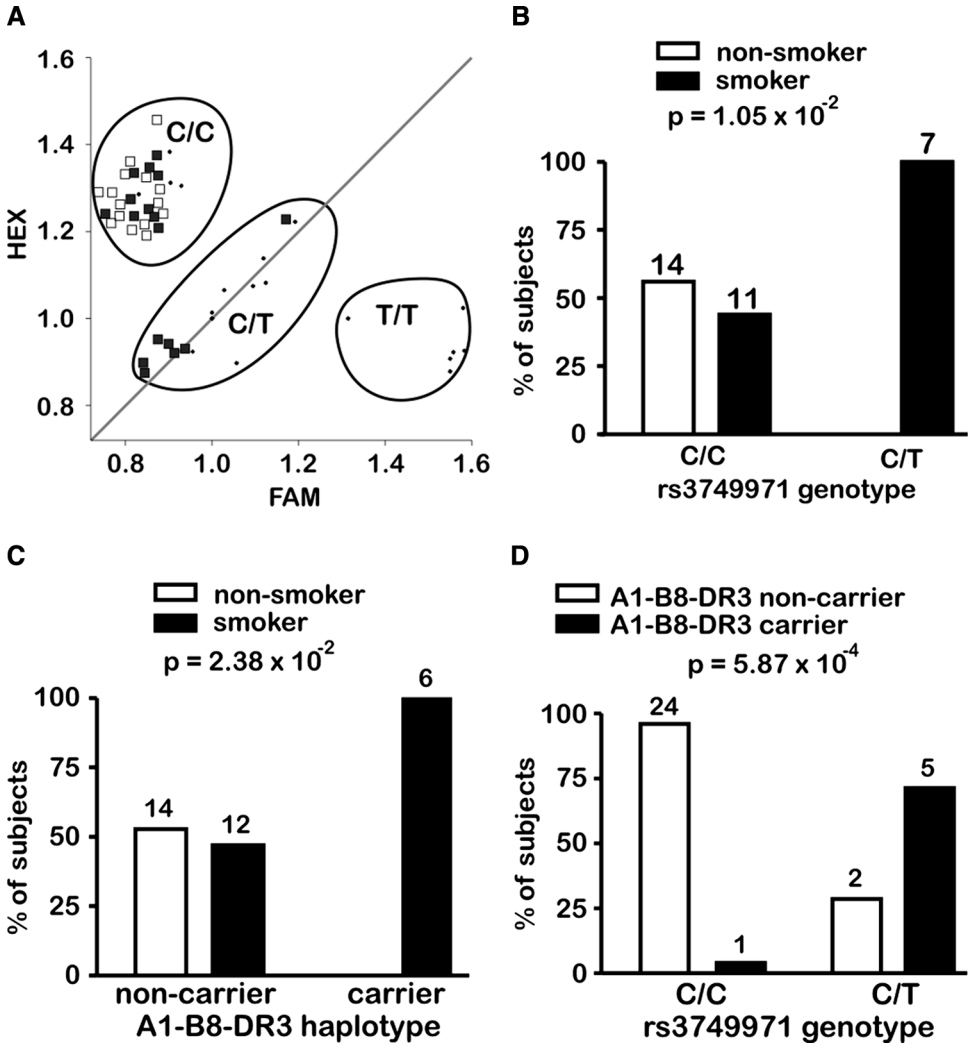

FIG. 3. Association of smoking behavior with the *rs3749971* genotype and the A1-B8-DR3 haplotype. (**A**) HEX and FAM are primer fluorophores marking the *rs3749971* T and the C allele primers, respectively. Values indicate the normalized relative number of PCR cycles necessary to reach the genotyping threshold for each allele. HEX values around 1 (or below) are indicative for the presence of the T allele, and the same is true for FAM values, indicating the presence of the C allele. Filled squares: 17 smokers; open squares: 15 nonsmokers; dots: controls. (**B**) Association of smoking behavior with *rs3749971* genotypes. (**C**) Association of smoking behavior with the carrier state of A1-B8-DR3. (**D**) Association of A1-B8-DR3 with *rs3749971* genotypes. (**B–D**) The number of individuals in each category is indicated above the corresponding bars, as well as the *p*-values, as determined by Fisher's exact test.

the ligand binding site. Keller *et al*. (2007) have recently demonstrated that a variant of an OR gene can substantially influence sensitivity (in both intensity and pleasantness) to specific odors in humans. They showed that a mutant allele of the *OR7D4* gene encoding an OR with two amino acid substitutions (residues 88 and 133) as compared to the most common allele causes functional impairment of the receptor *in vitro*. These substitutions also alter the perception of the smell of androstenone and androstadienone in a significant manner. Residues 88 and 133 are located within the first extracellular and the second intracellular loop. At least residue 88 is close to the beginning of the third transmembrane domain, suggesting that the region in the vicinity of residue 97 in the OR12D3 protein might indeed be involved in ligand binding. Further support for a role of amino acids close to the residue 97 in ligand binding is provided by the recently published crystal structure of the human β_2 adrenergic G-protein–coupled receptor (Rasmussen *et al.*, 2007).

Apart from social and psychological factors (Pomerleau *et al.*, 1992; Barman *et al.*, 2004; Lerer *et al.*, 2006), it has been shown that the individual genetic constitution plays a role in initiating and continuing tobacco consumption (Sullivan and Kendler, 1999; Li *et al.*, 2003). Genes within the xHLA contribute as well, as suggested by the finding of an HLA haplotype–dependent association of smoking in two independent European cohorts, with a clear-cut gender bias toward females (Füst *et al.*, 2004). The present study confirms these results by identifying an HLA-linked OR allele that is associated with tobacco abuse. In addition, our work implies that the rs3749971^T allele is involved in the smoking-induced aggravation of certain autoimmune diseases that can be observed in patients carrying A1-B8-DR3 (Hegediüs *et al.*, 2004; Holm *et al.*, 2005). Given the fact that the A1-B8-DR3 haplotype occurs with a frequency of 5–10% in Caucasians (Alper *et al.*, 2006), the *rs3749971* polymorphism should be suitable for large-scale screening tests, particularly among young people. Studies of the isolated OR12D3 protein will be indispensable to evaluate the consequences of the Thr97Ile exchange within this receptor on its ligand specificity.

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Disclosure Statement

The authors declare that no competing interests exist.

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