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Mutations in Olfactory Signal Transduction Genes Are Not a Major Cause of Human Congenital General Anosmia

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Abstract

Anosmia affects the western world population, mostly the elderly, reaching to 5% in subjects over the age of 45 years and strongly lowering their quality of life. A smaller minority (about 0.01%) is born without a sense of smell, afflicted with congenital general anosmia (CGA). No causative genes for human CGA have been identified yet, except for some syndromic cases such as Kallman syndrome. In mice, however, deletion of any of the 3 main olfactory transduction components (guanidine triphosphate binding protein, adenylyl cyclase, and the cyclic adenosine monophosphate–gated channel) causes profound reduction of physiological responses to odorants. In an attempt to identify human CGA-related mutations, we performed whole-genome linkage analysis in affected families, but no significant linkage signals were observed, probably due to the small size of families analyzed. We further carried out direct mutation screening in the 3 main olfactory transduction genes in 64 unrelated anosmic individuals. No potentially causative mutations were identified, indicating that transduction gene variations underlie human CGA rarely and that mutations in other genes have to be identified. The screened genes were found to be under purifying selection, suggesting that they play a crucial functional role not only in olfaction but also potentially in additional pathways.

Key words: anosmia, *CNGA2*, *GNAL*. *ADCY3*, linkage analysis, SNP

Introduction

Olfactory deficits have a strong effect on human quality of life, including compromised personal safety and eating disorders (Hummel and Nordin 2005). Studies in the western world suggest that ~1% of the human population suffers from olfactory disorders (Quint et al. 2001). Other studies show that as much as ~5% of all individuals above the age of 45 suffer from anosmia (Bramerson et al. 2004; Landis et al. 2004). Most of the affected people have an acquired condition, which develops during life, due to allergy, viral upper respiratory tract infection, nasal sinus diseases, head trauma, inhalation of noxious chemicals, or medicinal drug intake (Zusho 1982; Apter et al. 1999). A much smaller minority is born without a sense of smell, a rare condition. This may be isolated, appearing as the only deficit observed (Online Mendelian Inheritance in Man [OMIM] %107200), referred to here as congenital general anosmia (CGA). Alternatively, it is syndromic, appearing in conjunction with other deficits or anomalies, as exemplified by the well-studied Kallmann syndrome (OMIM +308700), where general anosmia, secondary to olfactory bulb agenesis, appears together with hypogonadotropic hypogonadism.

The prevalence of CGA has been roughly estimated as 1 in 10 000 (Quint et al. 2001; Temmel et al. 2002). Because there is no positive test for CGA, diagnosis has been based on the complete lack of the sense of smell for an entire lifespan, null performance in olfactory sensitivity tests, and the lack of

any alternative medical explanation. Using cranial magnetic resonance imaging (MRI) on CGA patients, morphological abnormalities have been shown to include hypoplastic or aplastic olfactory bulbs and hypoplasia in the olfactory tract in combination with shallow olfactory sulcus (Abolmaali et al. 2002). Biopsies of the olfactory region have found normal respiratory epithelium but total or partial lack of olfactory epithelium (Jafek et al. 1990; Leopold et al. 1992). Interestingly, the olfactory epithelium of 2 anosmic patients with Kallmann syndrome included functionally mature olfactory neurons, suggesting that they are able to differentiate in the absence of fully developed olfactory bulbs (Rawson et al. 1995).

Most of the CGA cases reported so far were sporadic, and only a few were familial. In a previous study that included 22 patients, 8 of them had a familial history of CGA (Leopold et al. 1992). Other sporadic cases have been described mostly in children and adolescents (Vowles et al. 1997; Assouline et al. 1998; Ho and Carrie 2001; Nishida et al. 2004). The published familial cases include a large 4-generation family with 27 affected individuals from the Faroe Islands (Lygonis 1969) and 8 American familial cases described by Leopold (Leopold et al. 1992). More recently, linkage analysis in 2 Iranian families with 9 affected members suggested a 46 cM linkage interval on chromosome 18 (Ghadami et al. 2004). Although the total number of familial CGA cases, reported so far, is small, all the published pedigrees suggest an autosomal dominant mode of inheritance with partial penetrance (Leopold et al. 1992; Ghadami et al. 2004).

To date, no causative gene has been described for isolated human CGA. For some syndromic cases, such as Kallmann and Bardet-Biedl syndromes (OMIM #209900), causative genes have been identified (Keith 1984; Franco et al. 1991; Dode et al. 2003). Interestingly, one mutation in *KAL1*, the gene responsible for the X-linked form of Kallmann syndrome, was reported to cause either hypogonadism and anosmia or isolated anosmia in 2 brothers (Parenti et al. 1995), making *KAL1* a suitable CGA candidate gene. The causative genes for the above-mentioned syndromes encode for developmental (Hardelin 2001) or morphology determining factors (Kulaga et al. 2004) but not for primary transduction pathway components.

In contrast, in a mouse model, inactivation of the 3 major transduction components in the primary sensory neurons revealed behavioral phenotypes consistent with general anosmia. The deleted components included a subunit of the olfactory cyclic nucleotide–gated cation channel (*Cnga2*) (Brunet et al. 1996), the alpha subunit of a stimulatory olfactory G-protein (*Gnal*) (Belluscio et al. 1998), and the cyclic adenosine monophosphate–generating enzyme adenylyl cyclase type III (*Adcy3*) (Wong et al. 2000).

In an attempt to further elucidate the genetic basis of CGA, we have collected 66 CGA families (83 affected subjects) and applied 2 complementary genetic approaches, whole-genome screen for the largest families and direct mutation detection for 3 candidate genes *GNAL*, *CNGA2*, and *ADCY3* in the entire cohort. We were able to identify neither a linkage interval for this phenotype nor mutations in the 3 transduction genes. We conclude that these 3 genes are not likely to constitute a major genetic basis for CGA.

Materials and methods

Subjects studied

All studies were conducted with the approval of The Institutional Review Board for human experiments in the Meir Hospital, Kfar Saba, Israel, protocol no. T15601. Participants in the present study signed a consent form. Potential anosmic cases were verified by the medical individual's history, physical examination, and olfactory sensitivity screening test. Determination of CGA in deceased persons was according to their relative's recollection. Forty-three anosmic males, 40 anosmic females, and 186 normal family members were recruited, and blood samples or buccal swabs were obtained. Additional control DNA samples were obtained from the National Laboratory for the Genetics of Israeli Populations at Tel-Aviv University (www.tau.ac.il/medicine/NLGIP/nlgip.htm).

Olfactory sensitivity screening

Olfactory sensitivity screening was carried out using a forced-choice 3-way bottle test (Gross-Isseroff et al. 1989, 1992; Fortier et al. 1991). We used 2 odorants (isoamyl acetate and Eugenol, 30 μl/ml) dissolved in mineral oil, avoiding excessively high concentrations of odorants that might lead to trigeminal perception. We used also Eugenol because isoamyl acetate can stimulate the trigeminal nerve at extremely high concentrations (Doty 1975; Doty et al. 1978). The use of both odorants assured that general anosmia would be missed only in individuals with unusually high trigeminal sensitivity for both of the odorants used. We note that only one individual was anosmic to Eugenol but responded correctly to isoamyl acetate, perhaps due to trigeminal stimulation.

For each odorant, 8 trials were conducted. On each trial, subjects were presented with one stimulus and 2 solvent-only blanks. Subjects were asked to sniff and choose (if necessary, guess) the different odor among the 3 samples. Subjects performing at chance level, guessing correctly less than 4 times, were considered anosmics.

Genome scan with markers and linkage analysis

Genomic DNA was extracted from whole blood, whenever available, otherwise buccal swabs were obtained. A genome scan using 400 microsatellite marker loci with an average distance of 10 cM (Linkage Mapping Set v2-MD10, Applied Biosystems, Foster City, CA) was performed, samples run on a DNA sequencer (Model 3100, Applied Biosystems) and allele calling assigned using the software Genescan and Genotyper.

The power to detect a linkage interval was calculated utilizing the slink and msim programs from the LINKAGE package software (http://linkage.rockefeller.edu) (Lathrop and Lalouel 1984; Lathrop et al. 1984), assuming an autosomal dominant inheritance with incomplete penetrance (90%) and a disease allele frequency of 0.001. Slink simulated 100 different genotypes for the given pedigrees under the assumption of linkage. The simulated genotypes were analyzed for linkage in each pedigree.

Two-point linkage analyses were performed using the software package LINKAGE as above, under the assumption of a dominant autosomal model with a partial penetrance of 80% or 90%. Allele frequency for each marker was set as $1/N$ (where N is the observed number of alleles).

Polymerase chain reaction amplification

Fragments covering the entire coding region and exon/intron boundaries of the 3 candidate genes *ADCY3* (NM_004036.2), *CNGA2* (NM_005140.1), and *GNAL* isoforms (NM_182978.1 and NM_002071.1) were amplified from genomic DNA samples. Two additional exons were predicted in the gene *CNGA2* using the partial mRNA AK128186 and the Genescan software (http://genes.mit.edu/GENSCAN.html) and amplified as above. Amplification primers were designed using Oligo software (Molecular Biology Insights, Inc., Cascade, CO) or Primer3 web server (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Primer sequences and polymerase chain reaction (PCR) conditions for each amplicon are summarized in Supplementary Table 1. Amplification was performed in a 50-μl reaction volume, using HotStart Taq polymerase (Qiagen, Hilden, Germany), under standard cycling conditions.

Single nucleotide polymorphism discovery by denaturing high-performance liquid chromatography

For single nucleotide polymorphism (SNP) discovery, 46 fragments, covering the coding regions and exon/intron boundaries of the 3 candidate genes, were analyzed by denaturing high-performance liquid chromatography (DHPLC, using Transgenomic Wave DNA fragment analysis system) (Bercovich and Beaudet 2003; Hasin et al. 2004). This method has been reported to have a sensitivity and specificity exceeding 96% (Xiao and Oefner 2001). In addition, its false-negative rate in several studies was 0 and its false-positive rate was 3% (Colosimo et al. 2002; Ravnik-Glavac et al. 2002; Wu et al. 2003).

The screening was performed in 64 unrelated anosmic individuals (128 chromosomes) and 3 normal control individuals (6 chromosomes) for the genes *ADCY3* and *GNAL*, and in 41 anosmic (41 chromosomes) males and one nonanosmic control (1 chromosome) for *CNGA2*. Nonsynonymous polymorphisms in the last gene were also screened in 48 normal unrelated individuals, 24 Ashkenazi Jews and 24 Sepharadi Jews. DNA alteration analysis was performed using a WAVE apparatus from Transgenomic Inc. (Omaha, NE). The PCR products were denatured at 95 °C for 5 min and cooled to 65 °C at a temperature gradient of 1 °C/min. The samples were kept at 4 °C until 5 μl were applied to a preheated C18 reversed-phase column based on nonporous poly (styrene-divinyl-benzene) particles (DNA-Sep Cartridge, CAT no. 450181; all DHPLC catalog numbers are from Transgenomic Inc.). DNA was eluted within a linear acetonitrile gradient consisting of buffer A (0.1 M triethylammonium acetate [TEAA], CAT no. SP5890) and buffer B (0.1 M TEAA, 25% acetonitrile, CAT no. 700001). Temperature of heteroduplex detection was deduced from the Transgenomic software (Wavemaker 4.2) and Stanford DHPLC melting program (http://insertion.stanford.edu/meltdoc.html), which analyzes the melting profile of the specific DNA fragment. All the found SNPs were submitted to dbSNP (http://www.ncbi.nlm.nih.gov/SNP/index.html), and their identifiers are NCBI_ss# 52084260-81.

DNA sequencing

For resequencing, PCR fragments were reamplified from the corresponding genomic DNA sample and subjected to direct sequencing using dye terminators. The sequencing reaction was performed at 56 °C, using PCR primers for both the forward and reverse strands. Sequence comparisons and SNP visualization were performed using the Sequencher software (Gene Codes Corporation, Ann Arbor, MI). Each SNP was identified in at least 2 independent PCR amplifications and appeared in at least 2 sequencing reactions.

Results

Population recruitment

The population recruitment was done by advertisements in major newspapers in Israel. A comprehensive examination to exclude cases of acquired general anosmia included an extensive questionnaire about the medical history of each subject, physical examination, and olfactory sensitivity testing. Following the examination, 83 CGA subjects within 66 families were recruited, the largest CGA cohort reported. Based on an estimate of direct and indirect readership of 2 million and of 50% response, this amounts to a CGA prevalence of 1 in ~10 000. This is in general agreement with previous estimates of 3% CGA among anosmics (Temmel et al. 2002), which in themselves are estimated as 1% in the general population (Quint et al. 2001). The ethnic segregation of the CGA subjects (40 females and 43 males) and number of affected individuals per family are presented in Figure 1 (see also Supplementary Table 2).

Linkage analysis

Whole-genome linkage analysis with 400 microsatellite markers was utilized in an attempt to identify genetic markers associated with anosmia. It was performed in families A002

(4 affected individuals) and A003 (5 affected individuals), assuming a dominant mode of inheritance. For these CGA families, such mode is more probable than a recessive one because there are affected individuals in every generation and the assumption of 3 carriers outside the nuclear families in each pedigree is considerably less parsimonious. Partial penetrance is suggested by the following observations (Figure 2):

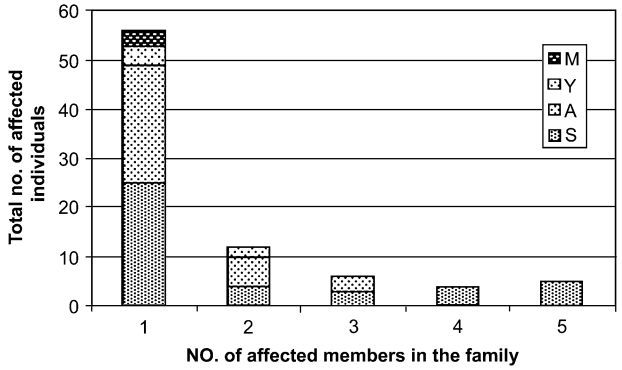

Figure 1 Ethnic and familial distribution of anosmic individuals. The exact numbers of affected individuals are detailed in Supplementary Table 1. M, Mixed ethnic group; Y, Yemenite Jews; A, Ashkenazi Jews; S, Sepharadi Jews.

1) An obligatory carrier was found to be healthy (Family A002, II-2); 2) a hyposmic individual is seen (Family A002, III-2); 3) in generation III of families A002 and A003, only 4 anosmics are seen out of 15 individuals whose parents were anosmics, considerably less than the expected 50%.

The power to detect a linkage interval in these families was calculated. The probability of reaching a logarithm of the odd (LOD) score of 3 or higher in family A002 was 11% and for family A003 was 33%. Although the power was very low, especially for family A002, the maximum expected LOD scores were 3.7 and 3.1 for families A002 and A003, respectively.

In a first round, genotypes were determined for partial cohorts (Figure 2). Two-point LOD scores were computed employing a dominant autosomal model, with partial penetrance of 80% and 90%. Each family was analyzed separately because CGA is presumed to be genetically heterogeneous, that is, the causative gene in each family could be different. The 9 markers with the highest LOD score values (all ranging between 1.0 and 1.5) were further analyzed in the second step, where the remaining family members, for whom only buccal swabs were available, were genotyped. The newly calculated LOD score values remained similar to the previous ones, (see Supplementary Table 3). Given the possibility that

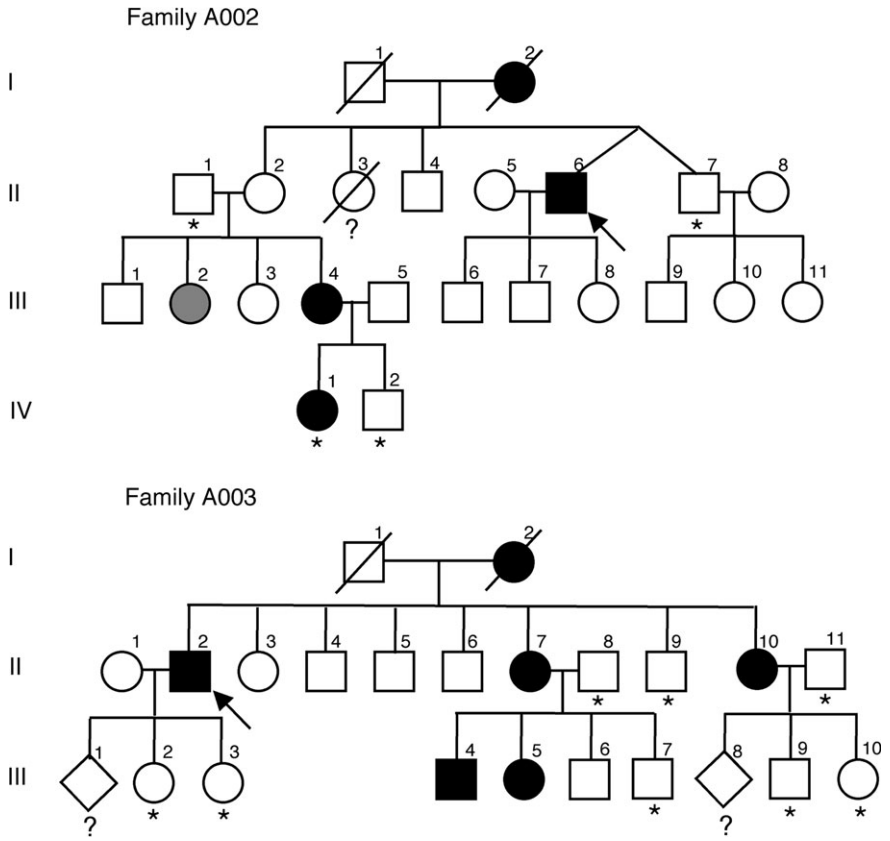

Figure 2 Pedigrees of 2 families with CGA. The arrows mark the probands. Square, male; circle, female; black, general anosmic; gray, general hyposmic; diamond, unknown gender; question mark, unknown phenotype; crossed, deceased, * indicates that only buccal swabs were available for these individuals. Note that partial pedigrees are drawn, offspring of healthy individuals from generation II are not always shown.

Table 1 A partial list of CGA candidate genes

Name	Symbol	Proposed role	Accession number	Chromosome	Coordinate	Ref
Gαolf[a]	*GNAL*	Transduction	NM_002071	18p11.21	11,742,185	1
Adenyl cyclase III[a]	*ADCY3*	Transduction	NM_004036	2p23.3	24,953,691	2
Cyclic nucleotide–gated channel α[a]	*CNGA2*	Transduction	NM_005140	Xq28	150,573,785	3
Cyclic nucleotide–gated channel β	*CNGB1*	Transduction	NM_001297	16q13	56,475,003	4
Cyclic nucleotide–gated channel α-like	*CNGA4*	Transduction	NM_001037329	11p15.4	6,216,910	4
Calcium/calmodulin-dependent protein kinase		Modulation				5
Subunit alpha	*CAMK2A*		NM_015981	5q32	149,579,247	
Subunit beta	*CAMK2B*		NM_001220	7p13	44,032,136	
Subunit gamma	*CAMK2G*		NM_001222	10q22.2	75,242,264	
Subunit delta	*CAMK2D*		NM_001221	4q26	114,733,168	
Calcium/calmodulin phosphodiesterase	*PDE1C*	Modulation	NM_005020	7p14.3	31,602,486	6
cAMP-activated phosphodiesterase	*PDE4A*	Modulation	NM_006202	19p13.2	10,424,636	7
Regulator of G- protein signaling 2	*RGS2*	Attenuation of ADCY3	NM_002923	1q31.2	189,509,827	8
Regulator of G- protein signaling 3	*RGS3*	G-protein regulation	NM_021106	9q32	113,422,493	9
G-protein–coupled receptor kinase 3	*ADRBK2*	Desensitization	NM_005160	22q12.1	24,285,499	10
Neural cell adhesion molecule	*NCAM2*	Axon fasciculation	NM_004540	21q21.1	21,292,503	11
Olfactory marker protein	*OMP*	Growth factor, modulation	NM_006189	11q13.5	76,491,533	12
Odorant-binding protein	*OBP2A*	Odorant binding	NM_014582	9q34.3	135,663,929	13
Cytochrome P-450	*CYP2G1*	Odorant clearance	AK127151	19q13.2	46,088,571	14
UDP glucosyl- transferase	*UGT2A1*	Odorant clearance	NM_006798	4q13.3	70,635,732	15
Arrestin2	*ARRB2*	Desensitization	NM_004313	17p13.2	4,560,537	16
Olf/early B-cell factor 1	*EBF*	Gene transcription	NM_024007	5q33.3	158,058,005	17
Olf/early B-cell factor 2	*EBF2*	Gene transcription	NM_022659	8p21.2	25,757,489	17
Olf/early B-cell factor 3	*EBF3*	Gene transcription	NM_001005463	10q26.3	131,523,536	17
Zinc finger protein 423 (olf1-associated)	*ZNF423*	Inhibition of Olf/EBF	NM_015069	16q12.1	48,082,114	18
Nuclear factor I	*NFIC*	Gene transcription	NM_005597	19p13.3	3,317,572	19
Kallmann syndrome 1 protein	*KAL1*	Olfactory bulb development	NM_000216	Xp22.31	8,306,650	20
Achaete-scute complex homolog-like 1	*ASCL1*	OE development	NM_004316	12q23.2	101,853,930	21
Hairy and enhancer of split 1	*HES1*	OE development	NM_005524	3q29	195,336,635	22
Paired box gene 6	*PAX6*	OE development	NM_000280	11p13	31,768,057	23

[a]The 3 major candidate genes.References: 1, (Belluscio et al. 1998); 2, (Bakalyar and Reed 1990); 3, (Brunet et al. 1996); 4, (Bonigk et al. 1999); 5, (Wei et al. 1998); 6, (Borisy et al. 1992); 7, (Borisy et al. 1993); 8, (Sinnarajah et al. 2001); 9, (Bruch and Medler 1996); 10, (Peppel et al. 1997); 11, (Yoshihara et al. 1997); 12, (Carr et al. 1998); 13, (Pelosi 2001); 14, (Nef et al. 1989); 15, (Lazard et al. 1990); 16, (Dawson et al. 1993); 17, (Wang et al. 1997); 18, (Tsai and Reed 1998); 19, (Behrens et al. 2000); 20, (Hardelin et al. 2000); 21, (Calof et al. 1998); 22, (Cau et al. 2000); 23, (Sisodiya et al. 2001).OE, olfactory epithelium; Ref, references; olf, olfactory.

the hyposmia of individual III-2 in family A002 (Figure 2) may have a different cause than the anosmia in the rest of the family, we performed the linkage analysis 3 times, defining individual III-2 as affected, unaffected, and of unknown phenotype. All calculated LOD scores were comparable.

In view of the following facts, 1) LOD scores did not improve when including in the analysis additional family members in the second step and 2) the highest LOD scores observed were supported by a single marker in each genomic location, we believe these LOD scores are not significant.

Combining the LOD scores of the 2 families resulted in maximum LOD scores around 1.7 because the maximum for each family correspond to different genomic locations. In addition, none of these 9 markers were located in the vicinity of potential CGA candidate genes (Table 1). Thus, the results of the genome scan in these 2 families could not define a linkage interval in any genomic location. This result is not surprising in view of the small size of the recruited CGA families and the observed partial penetrance that limited the power of the genetic analysis.

X-linked genetic analyses

Family A001 has 3 affected grandsons descendant from an affected maternal grandfather, hence an X-linked mode of transmission seemed possible, and linkage to chromosome X was tested in this family. Notably, 2 CGA candidate genes, *CNGA2* and *KAL1*, are located on chromosome X. We genotyped 18 microsatellites located along chromosome X in the 3 anosmic siblings and their mother and inferred haplotypes for the mother. Under the parsimonious assumption of no double recombination in 2 siblings, in the region of ~50 Mb or less in which the mother is homozygous for 3 contiguous microsatellites, no common haplotype for any region on chromosome X was found in the 3 anosmic siblings (Figure 3). This result excludes the hypothesis that CGA in family A001 might be caused by a gene located on chromosome X.

Mutation screening by denaturating chromatography in candidate genes

Candidate genes could include genes involved in olfactory development, in olfactory transduction and its termination, in higher levels of olfactory signal processing, and additional genes controlling their expression (Table 1). Some of the genes are considered to be olfactory specific, thus mutations in these components are expected to result in only a sensory deficit.

We performed direct mutation screening in and near exons of the 3 major CGA candidate genes, *CNGA2*, *ADCY3*, and *GNAL*. A total of 46 amplicons covering the coding regions and exon/intron boundaries of the 3 genes were screened by DHPLC (Figure 4). The amplicons of the autosomal genes, *ADCY3* and *GNAL*, were screened in 64 unrelated anosmic individuals and 3 normal control individuals, whereas the X-linked gene *CNGA2* was screened in 41 anosmic males and 1 control. An additional control group was added to the screening whenever necessary. Each of the distinct DHPLC elution pattern was verified by resequencing.

Twenty-two single nucleotide variations were found, of which 12 were in introns or UTRs. The remaining 10 variations were in exons, 6 synonymous and 4 nonsynonymous (Tables 2 and 3). Only 5 of the 22 SNPs were novel polymorphisms, and the rest are included in genomic SNP databases, 3 of them only in Celera Genomics and 14 in dbSNP (Figure 4). All the variations were excluded from being disease-causing mutations as they were found also in the controls. Although for most of the amplicons, the number of controls was small (6 chromosomes), the SNP allele frequencies in anosmic and controls were similar. Our inability to detect causative mutations indicates that for the present CGA cohort, the mutations frequency is less than 2.3% in the *ADCY3* and *GNAL* genes and less than 7.1% in the *CNGA2* gene (P value = 0.05).

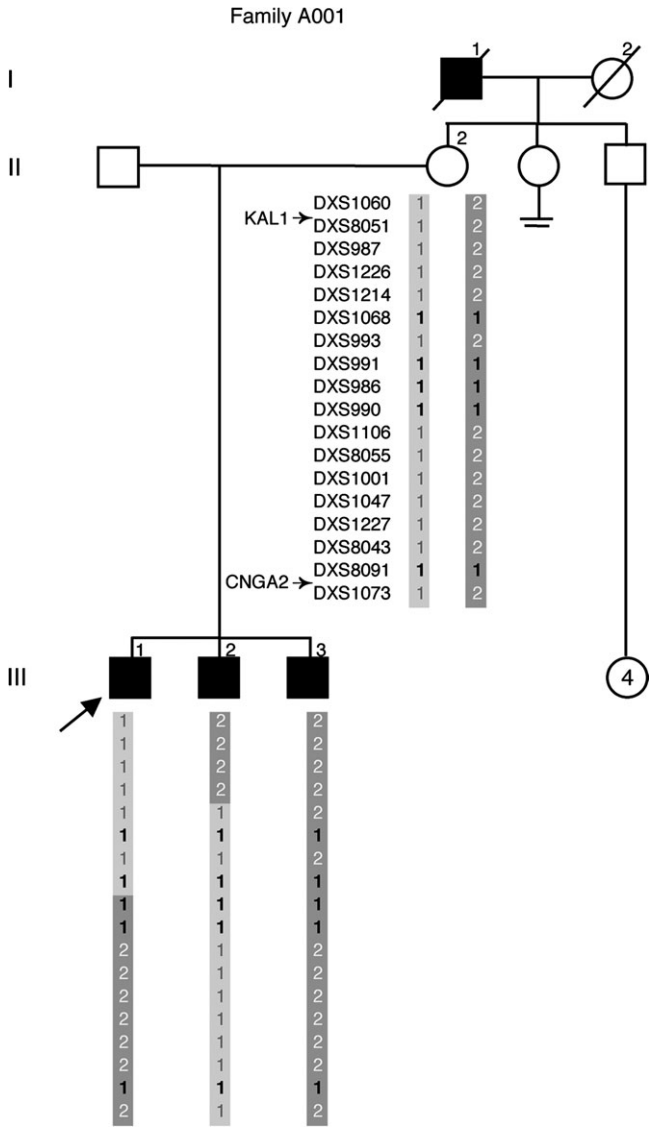

Figure 3 Pedigree of family A001, showing haplotypes on chromosome X. The arrow marks the proband. The haplotypes highlighted in yellow/light gray and green/dark gray are inferred from the offspring genotypes. The location of 2 candidate genes, *KAL1* and *CNGA2*, is shown by small arrows. Bold black letters represent homozygous microsatellites in II-2. Drawing features as in Figure 2.

Interestingly, the number of SNPs found in the 3 major candidate genes is lower than the number observed in a survey performed on 313 genes, where one SNP per 300 bp and one nonsynonymous SNP every 450 bp were detected

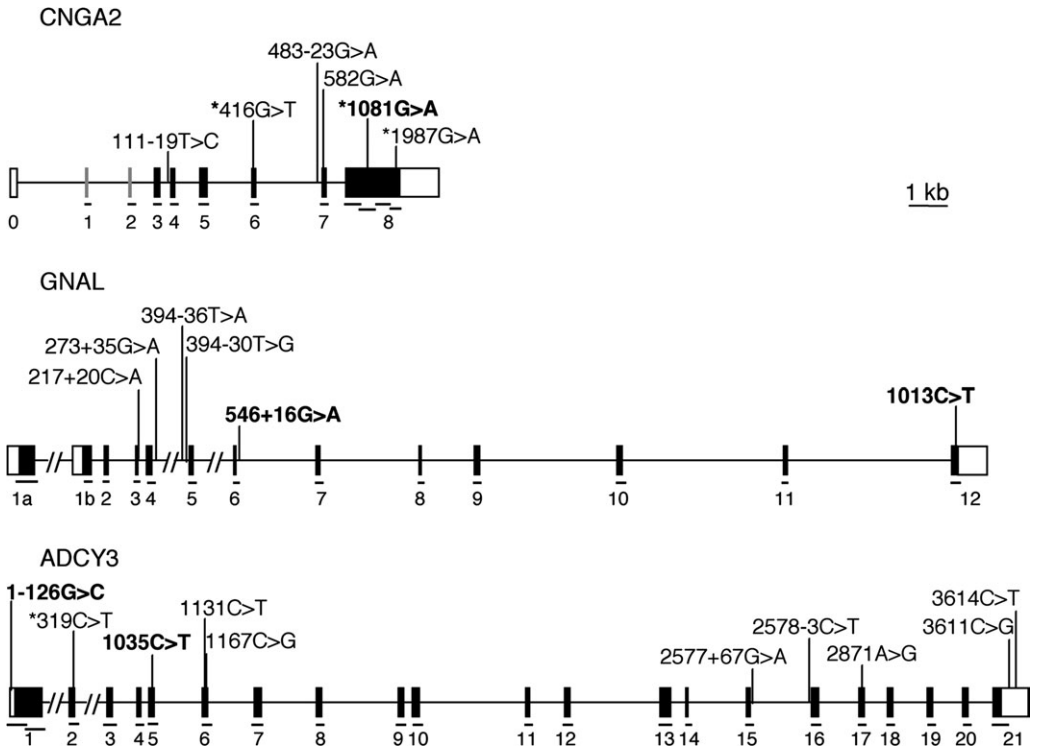

Figure 4 Polymorphisms and genomic organization of the 3 candidate genes. The exon–intron structure of the genes *CNGA2, GNAL*, and *ADCY3*, according to RefSeq (http://www.ncbi.nlm.nih.gov/RefSeq), is shown. Enumerated exons are depicted as boxes, solid for coding, empty for noncoding, and gray for predicted. The exon containing the initiator methionine is labeled 1, with 2 such alternatively spliced exons in *GNAL* (1a, 1b). SNPs are identified based on their position in the cDNA sequence, taking the A nucleotide of the ATG (the initiating codon) as the +1base. Nonsynonymous SNPs are marked with *. SNPs not present in genomic SNP databases, and reported here for the first time, are in bold. The 46 PCR amplicons used for DHPLC analysis, and fully covering all coding sequences, are represented by black lines under the exons.

in coding regions (Stephens et al. 2001; Salisbury et al. 2003; Pungliya et al. 2004) (Table 2).

Discussion

We described an effort to uncover the genetic basis of isolated CGA, a sensory deficit that has so far been only scantly analyzed genetically. We describe 66 CGA families that include 83 anosmic individuals, to our knowledge, the largest CGA cohort reported to date. Both the familial distribution of CGA individuals and the estimated prevalence were in agreement with previous studies (Leopold et al. 1992; Quint et al. 2001; Temmel et al. 2002). We found anosmia cases to be equally represented in all Jewish ethnic groups, and no significant differences were observed between males and females.

Other sensory deficits such as deafness and blindness had been much more extensively studied than CGA. They were typically found to be genetically heterogeneous and showed diverse modes of inheritance, including autosomal recessive, autosomal dominant, and X-linked (Bessant et al. 2001; Petit et al. 2001; Tekin et al. 2001), typically with full penetrance. Similarly, it is not unlikely that CGA would be a genetically heterogeneous condition, with different modes of inheritance for distinct causative genes and characteristically with full penetrance. However, a partial penetrance is observed here in the CGA pedigrees. This reduced penetrance has been previously noted in a 4-generation pedigree from Faroe Islands (Lygonis 1969) and 2 extended Iranian families (Ghadami et al. 2004). These repeated observations hint that CGA might not be an autosomal dominant affection, but an oligogenic one, requiring mutations in two or more loci. The

Table 2 Summary of DHPLC results

GENE	No. of exons	No. of amplicons	No. of SNPs in introns and UTRs	No. of SNPs in exons[a]	Coding SNPs/ 1 kb	Non-syn SNPs/ 1 kb
CNGA2	8	11	2	4 (3)	2.1	1.6
ADCY3	21	22	5	5 (1)	1.4	0.3
GNAL	13	13	5	1 (0)	1	0
313 genes[b]					3.4	2.2

[a]The numbers in parenthesis are nonsynonymous SNPs.
[b]The values for 313 genes are from (Stephens et al. 2001).syn, synonymus.

Table 3 Characterization of the nonsynonymous SNPs in the 3 major olfactory transduction genes

Gene	SNP identifier	Position in exon	Deduced amino acid change
CNGA2	416G>T	6	Trp140Leu
CNGA2	1081G>A	8	Val361Ile
CNGA2	1987G>A	8	Glu663Lys
ADCY3	319C>T	2	Pro107Ser

The SNP identifier and the exon number are defined as indicated in Figure 4.

failure to find a linkage interval in the CGA pedigrees could support an oligogenic model of inheritance but also be the result of an underpowered study. Oligogenic inheritance has been reported in other cases of disorders previously thought to be monogenic such as Bardet-Biedl syndrome and Hirschsprung disease (Badano and Katsanis 2002; Gabriel et al. 2002; Katsanis 2004; Van Heyningen and Yeyati 2004).

The 3 main olfactory transduction genes (*GNAL*, *CNGA2*, and *ADCY3*) were screened in the reported CGA cohort because they have been shown to be essential components of olfactory transduction in mice (Brunet et al. 1996; Belluscio et al. 1998; Wong et al. 2000). Mutations in other transduction genes have been related to a number of known inherited diseases, supporting a potential role for components of the olfactory transduction pathway in underlying CGA. Thus, G-protein, adenylyl cyclase, and calcium channel genes have been shown to be involved in developmental abnormalities of bone, hormone resistance and hormone hypersecretion, migraine, and several types of ataxia (Barrett et al. 1989; Abdel-Halim et al. 1998; Pietrobon 2002; Spiegel and Weinstein 2004). Likewise, mutations in each of the genes encoding visual transduction proteins may cause retinal dystrophy (Hims et al. 2003).

Mutations in the 3 major olfactory candidate genes do not seem to be a major cause of human CGA. Although regulatory regions were not screened, it is not likely that the whole anosmic cohort has mutations in regulatory but not in coding regions. Large deletions including one or more exons could disrupt one of the genes under study but would be not detectable by DHPLC.

The ratio of human–mouse divergence at nonsynonymous (amino acid replacement) sites versus synonymous sites (Ka/Ks $\ll 1$) (data not shown), together with the low number of SNPs observed in these genes, might hint that the genes are under purifying selection. Purifying selection might indicate that they have a conserved and vital function, olfaction related or otherwise. Examples for such nonolfactory vital functions might be the role of *Gnal* in the dopamine and adenosine receptor function in mouse basal ganglia (Corvol et al. 2001) and an association between promoter point mutations in *Adcy3* and decreased insulin release in a rat model of type 2 diabetes (Abdel-Halim et al. 1998).

Supplementary material

Supplementary Tables 1–3 can be found at http://www.chemse.oxfordjournals.org or at http://bioportal.weizmann.ac.il/HORDE/publications/CGA/.

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References

Abdel-Halim SM, Guenifi A, He B, Yang B, Mustafa M, Hojeberg B, Hillert J, Bakhiet M, Efendic S. 1998. Mutations in the promoter of adenylyl cyclase (AC)-III gene, overexpression of AC-III mRNA, and enhanced cAMP generation in islets from the spontaneously diabetic GK rat model of type 2 diabetes. Diabetes. 47:498–504.

Abolmaali ND, Hietschold V, Vogl TJ, Huttenbrink KB, Hummel T. 2002. MR evaluation in patients with isolated anosmia since birth or early childhood. Am J Neuroradiol. 23:157–164.

Apter AJ, Gent JF, Frank ME. 1999. Fluctuating olfactory sensitivity and distorted odor perception in allergic rhinitis. Arch Otolaryngol Head Neck Surg. 125:1005–1010.

Assouline S, Shevell MI, Zatorre RJ, Jones-Gotman M, Schloss MD, Oudjhane K. 1998. Children who can't smell the coffee: isolated congenital anosmia. J Child Neurol. 13:168–172.

Badano JL, Katsanis N. 2002. Beyond Mendel: an evolving view of human genetic disease transmission. Nat Rev Genet. 3:779–789.

Bakalyar HA, Reed RR. 1990. Identification of a specialized adenylyl cyclase that may mediate odorant detection. Science. 250:1403–1406.

Barrett D, Breslau NA, Wax MB, Molinoff PB, Downs RW Jr. 1989. New form of pseudohypoparathyroidism with abnormal catalytic adenylate cyclase. Am J Physiol. 257:E277–E283.

Behrens M, Venkatraman G, Gronostajski RM, Reed RR, Margolis FL. 2000. NFI in the development of the olfactory neuroepithelium and the regulation of olfactory marker protein gene expression. Eur J Neurosci. 12:1372–1384.

Belluscio L, Gold GH, Nemes A, Axel R. 1998. Mice deficient in G(olf) are anosmic. Neuron. 20:69–81.

Bercovich D, Beaudet AL. 2003. Denaturing high-performance liquid chromatography for the detection of mutations and polymorphisms in UBE3A. Genet Test. 7:189–194.

Bessant DA, Ali RR, Bhattacharya SS. 2001. Molecular genetics and prospects for therapy of the inherited retinal dystrophies. Curr Opin Genet Dev. 11:307–316.

Bonigk W, Bradley J, Muller F, Sesti F, Boekhoff I, Ronnett GV, Kaupp UB, Frings S. 1999. The native rat olfactory cyclic nucleotide-gated channel is composed of three distinct subunits. J Neurosci. 19:5332–5347.

Borisy FF, Hwang PN, Ronnett GV, Snyder SH. 1993. High-affinity cAMP phosphodiesterase and adenosine localized in sensory organs. Brain Res. 610:199–207.

Borisy FF, Ronnett GV, Cunningham AM, Juilfs D, Beavo J, Snyder SH. 1992. Calcium/calmodulin-activated phosphodiesterase expressed in olfactory receptor neurons. J Neurosci. 12:915–923.

Bramerson A, Johansson L, Ek L, Nordin S, Bende M. 2004. Prevalence of olfactory dysfunction: the skovde population-based study. Laryngoscope. 114:733–737.

Bruch RC, Medler KF. 1996. A regulator of G-protein signaling in olfactory receptor neurons. Neuroreport. 7:2941–2944.

Brunet LJ, Gold GH, Ngai J. 1996. General anosmia caused by a targeted disruption of the mouse olfactory cyclic nucleotide-gated cation channel. Neuron. 17:1–20.

Calof AL, Mumm JS, Rim PC, Shou J. 1998. The neuronal stem cell of the olfactory epithelium. J Neurobiol. 36:190–205.

Carr VM, Walters E, Margolis FL, Farbman AI. 1998. An enhanced olfactory marker protein immunoreactivity in individual olfactory receptor neurons following olfactory bulbectomy may be related to increased neurogenesis. J Neurobiol. 34:377–390.

Cau E, Gradwohl G, Casarosa S, Kageyama R, Guillemot F. 2000. Hes genes regulate sequential stages of neurogenesis in the olfactory epithelium. Development. 127:2323–2332.

Colosimo A, Guida V, De Luca A, Cappabianca MP, Bianco I, Palka G, Dallapiccola B. 2002. Reliability of DHPLC in mutational screening of beta-globin (HBB) alleles. Hum Mutat. 19:287–295.

Corvol JC, Studler JM, Schonn JS, Girault JA, Herve D. 2001. Galpha(olf) is necessary for coupling D1 and A2a receptors to adenylyl cyclase in the striatum. J Neurochem. 76:1585–1588.

Dawson TM, Arriza JL, Jaworsky DE, Borisy FF, Attramadal H, Lefkowitz RJ, Ronnett GV. 1993. Beta-adrenergic receptor kinase-2 and beta-arrestin-2 as mediators of odorant-induced desensitization. Science. 259:825–829.

Dode C, Levilliers J, Dupont JM, De Paepe A, Le Du N, Soussi-Yanicostas N, Coimbra RS, Delmaghani S, Compain-Nouaille S, Baverel F, et al. 2003. Loss-of-function mutations in FGFR1 cause autosomal dominant Kallmann syndrome. Nat Genet. 33:463–465.

Doty RL. 1975. Intranasal trigeminal detection of chemical vapors by humans. Physiol Behav. 14:855–859.

Doty RL, Brugger WE, Jurs PC, Orndorff MA, Snyder PJ, Lowry LD. 1978. Intranasal trigeminal stimulation from odorous volatiles: psychometric responses from anosmic and normal humans. Physiol Behav. 20:175–185.

Fortier I, Ferraris J, Mergler D. 1991. Measurement precision of an olfactory perception threshold test for use in field studies. Am J Ind Med. 20: 495–504.

Franco B, Guioli S, Pragliola A, Incerti B, Bardoni B, Tonlorenzi R, Carrozzo R, Maestrini E, Pieretti M, Taillon-Miller P, et al. 1991. A gene deleted in Kallmann's syndrome shares homology with neural cell adhesion and axonal path-finding molecules. Nature. 353:529–536.

Gabriel SB, Salomon R, Pelet A, Angrist M, Amiel J, Fornage M, Attie-Bitach T, Olson JM, Hofstra R, Buys C, et al. 2002. Segregation at three loci explains familial and population risk in Hirschsprung disease. Nat Genet. 31: 89–93.

Ghadami M, Morovvati S, Majidzadeh AK, Damavandi E, Nishimura G, Kinoshita A, Pasalar P, Komatsu K, Najafi MT, Niikawa N, et al. 2004. Isolated congenital anosmia locus maps to 18p11.23-q12.2. J Med Genet. 41:299–303.

Gross-Isseroff R, Ophir D, Bartana A, Voet H, Lancet D. 1992. Evidence for genetic determination in human twins of olfactory thresholds for a standard odorant. Neurosci Lett. 141:115–118.

Gross-Isseroff R, Ophir D, Marshak G, Ganchrow JR, Beizer M, Lancet D. 1989. Olfactory function following late repair of choanal atresia. Laryngoscope. 99:1165–1166.

Hardelin JP. 2001. Kallmann syndrome: towards molecular pathogenesis. Mol Cell Endocrinol. 179:75–81.

Hardelin JP, Soussi-Yanicostas N, Ardouin O, Levilliers J, Petit C. 2000. Kallmann syndrome. Adv Otorhinolaryngol. 56:268–274.

Hasin Y, Avidan N, Bercovich D, Korczyn A, Silman I, Beckmann JS, Sussman JL. 2004. A paradigm for single nucleotide polymorphism analysis: the case of the acetylcholinesterase gene. Hum Mutat. 24:408–416.

Hims MM, Diager SP, Inglehearn CF. 2003. Retinitis pigmentosa: genes, proteins and prospects. Dev Ophthalmol. 37:109–125.

Ho TP, Carrie S. 2001. Congenital anosmia. Int J Clin Pract. 55:418–419.

Hummel T, Nordin S. 2005. Olfactory disorders and their consequences for quality of life. Acta Otolaryngol. 125:116–121.

Jafek BW, Gordon AS, Moran DT, Eller PM. 1990. Congenital anosmia. Ear Nose Throat J. 69:331–337.

Katsanis N. 2004. The oligogenic properties of Bardet-Biedl syndrome. Hum Mol Genet. 13 Spec No 1:R65–R71.

Keith CG. 1984. Bardet-Biedl syndrome. Aust J Ophthalmol. 12:143–148.

Kulaga HM, Leitch CC, Eichers ER, Badano JL, Lesemann A, Hoskins BE, Lupski JR, Beales PL, Reed RR, Katsanis N. 2004. Loss of BBS proteins causes anosmia in humans and defects in olfactory cilia structure and function in the mouse. Nat Genet. 36:994–998.

Landis BN, Konnerth CG, Hummel T. 2004. A study on the frequency of olfactory dysfunction. Laryngoscope. 114:1764–1769.

Lathrop GM, Lalouel JM. 1984. Easy calculations of lod scores and genetic risks on small computers. Am J Hum Genet. 36:460–465.

Lathrop GM, Lalouel JM, Julier C, Ott J. 1984. Strategies for multilocus linkage analysis in humans. Proc Natl Acad Sci USA. 81:3443–3446.

Lazard D, Tal N, Rubinstein M, Khen M, Lancet D, Zupko K. 1990. Identification and biochemical analysis of novel olfactory-specific cytochrome P-450IIA and UDP-glucuronosyl transferase. Biochemistry. 29:7433–7440.

Leopold DA, Hornung DE, Schwob JE. 1992. Congenital lack of olfactory ability. Ann Otol Rhinol Laryngol. 101:229–236.

Lygonis CS. 1969. Familiar absence of olfaction. Hereditas. 61:413–416.

Nef P, Heldman J, Lazard D, Margalit T, Jaye M, Hanukoglu I, Lancet D. 1989. Olfactory-specific cytochrome P-450. cDNA cloning of a novel neuroepithelial enzyme possibly involved in chemoreception. J Biol Chem. 264: 6780–6785.

Nishida K, Kobayashi M, Adachi M, Nakamura S, Oishi M, Sakaida H, Imanishi Y, Majima Y. 2004. A case report on congenital anosmia. Nippon Jibiinkoka Gakkai Kaiho. 107:665–668.

Parenti G, Rizzolo MG, Ghezzi M, Di Maio S, Sperandeo MP, Incerti B, Franco B, Ballabio A, Andria G. 1995. Variable penetrance of hypogonadism in a sibship with Kallmann syndrome due to a deletion of the KAL gene. Am J Med Genet. 57:476–478.

Pelosi P. 2001. The role of perireceptor events in vertebrate olfaction. Cell Mol Life Sci. 58:503–509.

Peppel K, Boekhoff I, McDonald P, Breer H, Caron MG, Lefkowitz RJ. 1997. G protein-coupled receptor kinase 3 (GRK3) gene disruption leads to loss of odorant receptor desensitization. J Biol Chem. 272:25425–25428.

Petit C, Levilliers J, Hardelin JP. 2001. Molecular genetics of hearing loss. Annu Rev Genet. 35:589–646.

Pietrobon D. 2002. Calcium channels and channelopathies of the central nervous system. Mol Neurobiol. 25:31–50.

Pungliya MS, Salisbury BA, Nandabalan K, Stephens JC. 2004. Genetic variability and evolution of two pharmacologically important classes of genes. Pharmacogenomics. 5:115–127.

Quint C, Temmel AF, Schickinger B, Pabinger S, Ramberger P, Hummel T. 2001. Patterns of non-conductive olfactory disorders in eastern Austria: a study of 120 patients from the Department of Otorhinolaryngology at the University of Vienna. Wien Klin Wochenschr. 113:52–57.

Ravnik-Glavac M, Atkinson A, Glavac D, Dean M. 2002. DHPLC screening of cystic fibrosis gene mutations. Hum Mutat. 19:374–383.

Rawson NE, Brand JG, Cowart BJ, Lowry LD, Pribitkin EA, Rao VM, Restrepo D. 1995. Functionally mature olfactory neurons from two anosmic patients with Kallmann syndrome. Brain Res. 681:58–64.

Salisbury BA, Pungliya M, Choi JY, Jiang R, Sun XJ, Stephens JC. 2003. SNP and haplotype variation in the human genome. Mutat Res. 526:53–61.

Sinnarajah S, Dessauer CW, Srikumar D, Chen J, Yuen J, Yilma S, Dennis JC, Morrison EE, Vodyanoy V, Kehrl JH. 2001. RGS2 regulates signal transduction in olfactory neurons by attenuating activation of adenylyl cyclase III. Nature. 409:1051–1055.

Sisodiya SM, Free SL, Williamson KA, Mitchell TN, Willis C, Stevens JM, Kendall BE, Shorvon SD, Hanson IM, Moore AT, et al. 2001. PAX6 haploinsufficiency causes cerebral malformation and olfactory dysfunction in humans. Nat Genet. 28:214–216.

Spiegel AM, Weinstein LS. 2004. Inherited diseases involving g proteins and g protein-coupled receptors. Annu Rev Med. 55:27–39.

Stephens JC, Schneider JA, Tanguay DA, Choi J, Acharya T, Stanley SE, Jiang R, Messer CJ, Chew A, Han JH, et al. 2001. Haplotype variation and linkage disequilibrium in 313 human genes. Science. 293:489–493.

Tekin M, Arnos KS, Pandya A. 2001. Advances in hereditary deafness. Lancet. 358:1082–1090.

Temmel AF, Quint C, Schickinger-Fischer B, Klimek L, Stoller E, Hummel T. 2002. Characteristics of olfactory disorders in relation to major causes of olfactory loss. Arch Otolaryngol Head Neck Surg. 128:635–641.

Tsai RY, Reed RR. 1998. Identification of DNA recognition sequences and protein interaction domains of the multiple-Zn-finger protein Roaz. Mol Cell Biol. 18:6447–6456.

Van Heyningen V, Yeyati PL. 2004. Mechanisms of non-Mendelian inheritance in genetic disease. Hum Mol Genet. 13 Spec No 2: R225–R233.

Vowles RH, Bleach NR, Rowe-Jones JM. 1997. Congenital anosmia. Int J Pediatr Otorhinolaryngol. 41:207–214.

Wang SS, Tsai RY, Reed RR. 1997. The characterization of the Olf-1/EBF-like HLH transcription factor family: implications in olfactory gene regulation and neuronal development. J Neurosci. 17:4149–4158.

Wei J, Zhao AZ, Chan GC, Baker LP, Impey S, Beavo JA, Storm DR. 1998. Phosphorylation and inhibition of olfactory adenylyl cyclase by CaM kinase II in neurons: a mechanism for attenuation of olfactory signals. Neuron. 21:495–504.

Wong ST, Trinh K, Hacker B, Chan GC, Lowe G, Gaggar A, Xia Z, Gold GH, Storm DR. 2000. Disruption of the type III adenylyl cyclase gene leads to peripheral and behavioral anosmia in transgenic mice. Neuron. 27: 487–497.

Wu G, Hua L, Zhu J, Mo QH, Xu XM. 2003. Rapid, accurate genotyping of beta-thalassaemia mutations using a novel multiplex primer extension/denaturing high-performance liquid chromatography assay. Br J Haematol. 122:311–316.

Xiao W, Oefner PJ. 2001. Denaturing high-performance liquid chromatography: a review. Hum Mutat. 17:439–474.

Yoshihara Y, Kawasaki M, Tamada A, Fujita H, Hayashi H, Kagamiyama H, Mori K. 1997. OCAM: a new member of the neural cell adhesion molecule family related to zone-to-zone projection of olfactory and vomeronasal axons. J Neurosci. 17:5830–5842.

Zusho H. 1982. Posttraumatic anosmia. Arch Otolaryngol. 108:90–92.

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