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Erythrocyte Membrane Antigen Frequencies in Patients with Type II Congenital Smell Loss

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Abstract

OBJECTIVE—The objective of this study was to determine whether there are genetic factors associated with Type II congenital smell loss.

STUDY DESIGN—The expression frequencies of 16 erythrocyte antigens among patients with Type II congenital smell loss were determined and compared to those of a large control group.

METHODS—Blood samples were obtained from 99 patients with Type II congenital smell loss. Presence of the erythrocyte surface antigens A, B, M, N, S, s, Fy^a, Fy^b, D, C, c, E, e, K, Jk^a, and Jk^b was analyzed by blood group serology. Comparisons of expression frequencies of these antigens were made between the patients and a large control group.

RESULTS—Patients tested for the Duffy b antigen (Fy^b haplotype) exhibited a statistically significant 11% decrease in expression frequency compared to the controls. There were no significant differences between patients and controls in the expression frequencies for all other erythrocyte antigens (A, B, M, N, S, s, Fy^a, D, C, c, E, e, K, Jk^a, or Jk^b).

CONCLUSIONS—These findings describe the presence of a previously unrevealed genetic tendency among patients with Type II congenital smell loss related to erythrocyte surface antigen expression. The deviation in expression rate of Duffy b suggests a target gene and chromosome region in which future research into this form of congenital smell loss may reveal a more specific genetic basis for Type II congenital smell loss.

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Keywords

congenital smell loss; hyposmia; anosmia; Duffy; Duffy b; Fy^b; DARC

Introduction

Congenital smell loss is a condition characterized by inability to detect or recognize odors since birth and affects about 500,000 individuals in the US [1]. In about 12% of cases (Type I), the condition is familial and occurs in conjunction with other severe congenital neurological defects and somatic abnormalities including hypogonadotropic hypogonadism [2,3,4,5,6,7]. Multiple genetic abnormalities have been associated with these patients [7,8,9,10]. The remaining 88% of congenital smell loss cases (Type II) occur without gonadal and somatic abnormalities, usually without familial association; no genetic abnormalities have been associated with Type II patients [2].

Type I congenital smell loss was first reported in 1856 [11]. In the 20th century, it was further characterized by Kallmann³ and later De Morsier [12]. Kallmann syndrome patients exhibit hypogonadotropic hypogonadism, anosmia/hyposmia, color blindness, and other X-linked congenital abnormalities; they also exhibit low levels of sex and gonadotropin hormones, and suffer from absent or severely diminished smell function [13]. Patients with Type I congenital smell loss demonstrate a hereditary pattern, but they appear sporadically. They are the result of a complex genetic-environmental relationship probably involving oligogenic inheritance in many cases. Most of the responsible mutations are in genes that code for G-protein coupled receptors (GPCR) and their ligands. They appear to cause the primary developmental defects observed. Smell loss has been demonstrated to be related to anatomical changes mediated in part by these biochemical abnormalities [5,13,14]. Indeed magnetic resonance (MR) imaging of patients with Type I congenital smell revealed severe malformation or complete agenesis of key brain olfactory structures formed *in utero* including aplasty or hypoplasia of the olfactory bulbs and insufficient deepening of the olfactory sulci [2,15,16,17,18].

The pathogenesis of smell loss in patients with Type I congenital smell loss is heterogeneous. A model involving the failure of gonadotropin-releasing hormone (GnRH) synthesizing neurons to properly direct the morphogenesis of the brain structures has been established [19,20]. The failure of the neurite-directing protein anosmin-1 has also been implicated [21]. Various olfactory epithelial defects have also frequently been found in these patients including its absence, diminished size, and histological/anatomical abnormalities [22,23,24].

Type II congenital smell loss was first described nearly 100 years ago [18] and since then others have reported patients who display no clinical abnormalities other than congenital decreased or absent response to olfactory stimuli [2,22,23]. A family history of olfactory or gustatory disorders has usually not been discovered among these patients [2,25,26]. Genetic screening of several dozen individuals affected by Type II congenital smell loss in which a familial trait was present revealed no association between the disease state and mutations of 3 key olfactory signal transduction genes: cyclic nucleotide-gated channel alpha 2

(CNGA2), adenylate cyclase-stimulating G alpha protein, olfactory type (GNAL), and adenylyl cyclase type III (ADCY3) [27]. To date, genetic factors that might be associated with Type II congenital smell loss have remained elusive and most of the biology of the condition is still unknown.

MR imaging of Type II congenital smell loss patients revealed a heterogeneous group of olfactory CNS abnormalities including hypoplasia of olfactory bulbs, grooves, and sulci [26]. However, while frequent, olfactory CNS abnormalities in Type II congenital smell loss patients were not as common or as severe as those observed in Type I congenital smell loss patients; anatomical components of the normal olfactory system were found present in each patient [26].

The purpose of the present study is to investigate a possible basis for genetic changes that might be present in patients with Type II congenital smell loss. To do this we determined the erythrocyte membrane antigens in these patients and compared their rates of occurrence to those expected based on published control data.

We have collected blood samples for erythrocyte antigen analysis in patients with congenital smell loss beginning in 1980. Although more comprehensive techniques for studying genetic factors in disease are currently available, these techniques were not available at the time we initiated these studies. Thus, we studied erythrocyte antigen expression frequencies in these patients in an attempt to obtain some genetic information among these patients. By analyzing the expression frequencies of 16 antigens in these patients, we were able to obtain some information about their genomes and to determine whether some aspect of specific genes might be expressed similarly or in a different manner to a large control group. Statistically significant deviations in erythrocyte antigen expression frequencies between the patients and the control group would indicate a probability that the condition might be genetically associated. This technique has previously been employed to study genetic factors present in patients suffering from chronic kidney disease [28].

The antigens A and B (of the ABO blood group), M, N, S, and s (of the MNS blood group), Fy^a and Fy^b (of the Duffy blood group), D (of the RhD blood group), C, c, E, and e (of the RhCE blood group), K (of the Kell blood group), and Jk^a and Jk^b (of the Kidd blood group) were chosen for study because they exhibit a wide range of expression frequencies among a control group and have reliable serology techniques for their detection [29,30]. Although the antigens and the genes responsible for their expression may not be directly involved with olfaction or gustation, analysis of their expression patterns among the congenital smell loss patients may reflect a manifestation of genetic tendencies in this group of patients.

Materials and Methods

Patients

Ninety-nine consecutive patients [aged 6–70; 30±16y (mean±SD)] who reported lifelong smell loss without associated somatic abnormalities or hypogonadism presented at The Taste and Smell Clinic in Washington D.C. between 1980 and 2012 for evaluation and treatment of their smell loss. They were 44 men and 55 women. All patients who presented to The

Clinic with these clinical initial findings were included in this study. All exhibited normal growth, gonadal function and development for their age. All prepubescent children exhibited normal age related growth and development. None reported any family history of olfactory disorders. Those with children reported no sensory disorders in their offspring. A physical examination of each patient's head and neck revealed no clinical abnormalities. Magnetic resonance imaging of brain was performed in many of these patients. Olfactory structures were present in each patient studied and were consistent with findings demonstrated in patients with Type II congenital smell loss [2,26].

Clinical Assessment of Taste and Smell Function

Comprehensive evaluation of the patients' taste and smell function including measurements of detection and recognition thresholds and magnitude estimation were performed in accordance with the gustometry and olfactometry protocols described previously [31]. All patients exhibited abnormalities in smell function as measured by olfactometry [31]. These abnormalities were manifested by increased detection and/or recognition thresholds (decreased sensitivity) and decreased magnitude estimation (decreased sensitivity) for one or more odors (pyridine, nitrobenzene, thiophene and amyl acetate) as previously described [31].

Blood Processing

Blood was collected via venipuncture and immediately refrigerated. Within one to five days, red blood cell samples tested for blood group antigens.

Immunohematology

The patients' blood group antigens were tested with standard blood group serology methods [32].

Statistical Analysis

Comparisons of patient erythrocyte antigen frequencies were made to the expected antigen frequencies of a large control group consisting of several thousands of patients [29,30]. Expected antigen frequencies for the control group were obtained from The Blood Group Antigen FactsBook [29] and Blood Groups and Red Cell Antigens [30]. These published antigen expression frequencies used as controls for the present statistical analyses are based on many different peer-reviewed studies involving a large numbers of subjects in whom these measurements have been made over several decades. The data we present now reflect a comparison of results from this large control group. Deviations from the results of this control group indicate a statistical likelihood that the patient group is different from the large group of controls with regard to the probability of occurrence of the tested antigen expression frequencies.

Significance of difference was measured by the non-cumulative (exact) binomial test with $p < 0.05$ considered significant. The binomial test was deemed the most appropriate method to compare the number of instances of a binary phenomenon occurring within a tested group (e.g., the number of patients expressing a blood group antigen among all tested patients) to an expected probability of the phenomenon occurring (e.g., the frequency of individuals in

the control group who express the antigen). A statistically significant deviation indicates that results in the tested group differ significantly from that in the control group with regard to the probability of the occurrence of this phenomenon.

Since multiple comparisons were made between the patient and the control data, the Bonferroni-Holm method was applied to the threshold of significance to counteract familywise error [33]. Given that 16 comparisons were made (one for each antigen) and a single-comparison significance threshold of $p < 0.05$, the Holm-Bonferroni adjusted significance threshold became $p < 0.003125$ ($0.05/16$) for the test resulting in the lowest p value. If significance were determined for the first test, a significance threshold of $p < 0.003333$ ($0.05/15$) was applied to the test resulting in the next lowest p threshold. If significance were determined for the second test, a significance threshold of $p < 0.003571$ ($0.05/14$) was applied to the test resulting in the third lowest p threshold. This process stops once the first test result is observed, which exceeds the adjusted p threshold.

Institutional Review

This study was approved by the Institutional Review Board of the Georgetown University Medical Center, Washington D.C. All patients formally consented to participate in this study.

Results

Sixteen blood group antigens were tested in each patient with Type II congenital smell loss. Only the Duffy b antigen showed a statistically significant difference based on the Bonferroni-Holm correction for multiple statistical testing ($n=16$, $p < 0.003125$) (Table I).

Duffy Blood Group

For the two major Duffy blood group antigens (Table I), Fy^b expression frequency was significantly decreased relative to the control group; 99 patients were tested for Fy^b and only 72% ($n=71$) tested positive (versus 83% in the control group, $P=0.002$). However, there were no significant differences for Fy^a ; 64% ($n=63$) tested positive (versus 66% in the control group, $P=0.074$).

Analysis of the patients' Duffy phenotypes revealed that the diminished expression frequency of the Fy^b haplotype reflect a significant increase in the frequency of the Fy (a+b-) phenotype of roughly equal magnitude to the significant decrease in the frequency of the Fy (a+b+) phenotype. Of the patients tested for the Fy (a+b+) phenotype, 35% ($n=35$) tested positive (versus 49% in the control group, $P=0.002$) which represents a 14% decrease in frequency. Of the patients tested for the Fy (a+b-) phenotype, 28% ($n=28$) tested positive (versus 17% in the control group, $P=0.002$) which represents an 11% increase in frequency. Fy (a-b+), Fy (a-b-) phenotypes were expressed at frequencies that did not differ significantly from control group frequencies.

Other Blood Groups

None of the 15 other blood group antigens showed statistically significant differences based on the Bonferroni-Holm corrected p threshold (Table II).

Discussion

Most erythrocyte antigen expression frequencies in Type II congenital smell loss patients were consistent with those determined in a large control group. The major haplotypes and phenotypes of the ABO, MNS, RhD, RhCE, Kell, and Kidd blood groups were all expressed in the hyposmic patients at frequencies that did not differ from those found in the control group with regard to the probability of their expression. However, the antigen Fy^b was expressed at a significantly lower frequency ($p=0.00183$); this observation indicates that patients with Type II congenital smell loss are significantly different from the control group with regard the probability of expression of this haplotype and its associated phenotypes.

These data suggest that there are genetic factors present in patients with Type II congenital smell loss that distinguish them from the general population. If Type II congenital smell loss were a non-genetic syndrome, then we might expect the tested patients' erythrocyte antigen expression frequencies to match those of the controls. The statistically significant deviation in the expected frequency of the Duffy b haplotype indicates that Type II congenital smell loss patients as a group are not genetically representative of the controls. Since the amino acid and nucleotide sequence of the Duffy biochemical determinant is responsible for its antigenic state [29], these data suggest the presence of atypical allele frequencies in these patients [34].

The Duffy antigen receptor of chemokines (DARC) is the biochemical determinant of the Duffy blood group and belongs to a class of atypical chemokine receptors. The seven-pass transmembrane glycoprotein is expressed primarily on the membranes of erythrocytes and postcapillary venule endothelial cells as well as in a variety of other tissues. DARC features an active chemokine-binding pocket, capable of promiscuously binding about 20 proinflammatory chemokine ligands of the CC and CXC families [35]. Despite superficially resembling a classic G protein-coupled receptor, DARC completely lacks the DRY motif required for G-protein coupled signal transduction [36]. Although DARC cognate ligand binding induces none of the intracellular responses typical of GPCRs, it does induce internalization of the DARC-ligand complex that promotes chemokine transcytosis, patterning, and presentation [37].

The activity of DARC is not fully understood. It is possible to hypothesize that its chemokine handling functions extend to several signaling molecule classes, including cytokines. Indeed, pro-inflammatory and anti-inflammatory cytokines and adenylyl cyclase in nasal mucus have been implicated in the maintenance of normal olfactory receptor function [38]. Many patients suffering from smell loss (including Type II congenital smell loss) exhibited abnormalities in the balance of these cytokines [38] and in the concentration of adenylyl cyclase [39,40,41,42,43]. Treatment of these patients (including those with Type II congenital smell loss) with phosphodiesterase (PDE) inhibitors demonstrated improvement in smell function in about 50% of patients [44,45]. These drugs increase the

concentration of cAMP and cGMP (downstream messengers of adenylyl cyclase) in nasal mucus and help promote growth of olfactory receptors [46].

The Fy^b haplotype is overrepresented in patients suffering from graft-versus-host disease possibly due to more effective chemokine transcytosis and leukocyte recruitment mediated by that form of DARC [47]. Perhaps Fy^b is better at maintaining certain critical proinflammatory cytokine moieties in nasal mucus than Fy^a and smell loss associated with Type II congenitals is due in part to their lower incidence of Fy^b expression. Future research can investigate how this atypical receptor is implicated in this process.

The DARC gene (1q21-22), which codes for the Duffy antigen, is located in the center of a 1 Mbp region (158,700K – 159,700K bp) containing 18 identified olfactory receptor genes and pseudogenes [48,49]. Mutations of individual olfactory receptor genes could be responsible for specific anosmias (inability to sense specific classes of odorant stimuli) but probably not the general hyposmia seen in Type II congenital smell loss. However our understanding of how the CNS interprets input from individual olfactory receptors is still unclear, so we cannot rule out that specific receptor genes are implicated in congenital smell loss.

Smell loss inducing mutations in these surrounding genes in a common ancestor may have been passed down to the patients in this study. The ancestor's Duffy genotype would have tended to be preserved with the olfactory receptor gene mutations as a heritable packet over generations due to its proximity; the abnormal expression frequency of the Duffy b haplotype and the presence of congenital smell loss in this study's patients may therefore be a common effect of inheriting this ancestral chromosome region mostly intact.

We concluded that Type II congenital smell loss patients, as a group, are genetically distinct from individuals without this condition with regard to a certain haplotype expression frequency. Future research should seek to investigate the DARC gene and its gene locus on the long arm of chromosome 1 in Type II congenital smell loss patients. Investigation of the regulatory sequences and coding sequences that flank this gene on the chromosome should also be undertaken to determine if those are smell loss factors.

There are limitations to our study. Comparison of erythrocyte antigen expression frequencies was made with respect to a large control group about whom we have no specific knowledge with regard to any clinical abnormalities. However, results of the present study indicate that the frequency of Duffy b antigen expression in patients with Type II congenital smell loss is significantly different from that of the controls.

While a positive reaction with an antibody is sufficient to demonstrate the genetic sequence at one point, a negative antibody reaction is not sufficient to establish a precise genetic data point. For instance, the presence of a nucleotide other than A at base pair 125 of the DARC gene will alter the amino acid at position 42 and prompt a negative serological result for Fy^b; however, it is also possible that a patient with an A residue at base pair 125 yields a negative result. A mutation in a regulator, posttranslational modifying protein, etc. may result in a phenotype in which erythroid membranes lack the antigen despite the gene in question being of the positive-associated allelic variety. Moreover, a mutation resulting in a

substantial structural change to DARC may prevent binding by anti-Fy^b despite the presence of the native Asp-42. A negative immunoassay result also still allows that the protein may be expressed elsewhere as is seen in the erythroid silent phenotype of Duffy in African populations.

Comparison of the differences of these erythrocyte antigen expression frequencies allows for a narrow view into the genomes of these hyposmic patients. Initially we considered it unlikely that any deviations from expected frequencies would be found since we are only able to study an extremely small percentage of the total coding genome through erythrocyte antigen immunoassays. Indeed many similarities were found. Nevertheless, an abnormality was also discovered. Future research are needed to confirm the hypotheses we have described.

Conclusions

Patients with Type II congenital smell loss express the Duffy b erythrocyte antigen at a significantly lower frequency compared to a large control group. The abnormality in DARC expression suggests that there are genetic factors that may be associated with this disease and that 1q21-22 is a potential locus for one or more of these factors.

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Table I

Duffy Erythrocyte Blood Group in the General Caucasian Population and in Caucasian Patients with Type II Congenital Smell Loss

	Haplotypes		Phenotypes			
	Fya	Fyb	Fy(a+b+)	Fy(a+b-)	Fy(a-b+)	Fy(a-b-)
<i>a</i>	# Tested	99	99	99	99	99
<i>b</i>	# Positive	63	35	28	36	0
<i>c</i>	Control %	66%	49%	17%	34%	0%
<i>d</i>	Patient %	64%	35%	28%	36%	0%
<i>e</i>	P	0.074	0.002	0.002	0.074	1.000

a) Total number of Caucasian patients with Type II congenital smell loss tested for erythrocyte antigens Fya and Fyb

b) Number of patients among those tested for the antigens who tested positive

c) Frequency of antigens reported among the general population of Caucasians (Reid, 2012)

d) Frequency of antigens measured among patients

e) Probability that the differences between the patient and expected frequencies is significant as calculated by binomial test ($P < 0.003125$ is significant)

Table II
Erythrocyte Blood Groups in the General Caucasian Population and in Caucasian Patients with Type II Congenital Smell Loss

	ABO				MNS				RhCE				Kell		Kidd	
	A	B	O	AB	M	N	S	s	D	C	c	E	e	K	Jka	Jkb
a	# Tested	98	98	98	54	54	94	99	98	99	99	99	96	99	99	99
b	# Positive	40	12	41	5	35	41	92	83	73	79	23	95	11	72	75
c	Control %	43 %	9 %	44 %	4 %	72 %	55 %	89 %	85 %	68 %	80 %	29 %	98 %	9 %	77 %	74 %
d	Patient %	41 %	12 %	42 %	5 %	65 %	44 %	93 %	85 %	74 %	80 %	23 %	99 %	11 %	73 %	76 %
e	P	0.074	0.069	0.074	0.156	0.058	0.007	0.064	0.111	0.042	0.099	0.041	0.282	0.098	0.055	0.086

- a) Total number of Caucasian patients with Type II congenital smell loss tested for erythrocyte antigens
- b) Number of patients among those tested for the antigens who tested positive
- c) Frequency of antigens reported among the general population of Caucasians (Reid, 2012)
- d) Frequency of antigens measured among patients
- e) Probability that the differences between the patient and expected frequencies is significant as calculated by binomial test ($P < 0.003125$ is significant)