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Founder mutation(s) in the *RSPH9* gene leading to primary ciliary dyskinesia in two inbred Bedouin families

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Abstract

A rare mutation in the *RSPH9* gene leading to Primary Ciliary Dyskinesia was previously identified in two Bedouin families, one from Israel and one from the United Arab Emirates (UAE). Herein we analyze mutation segregation in the Israeli family, present the clinical disease spectrum, and estimate mutation age in the two families. Mutation segregation was studied by restriction fragment length analysis. Mutation ages were estimated using a model of the decrease in the length of ancestral haplotypes. The mutations in each of the two families had a common ancestor less than 95 and 17 generations in the past. If the mutations in the two families are descended from a common ancestor, that mutation would have to have arisen at least 150 generations ago. If the Bedouin population has been roughly constant in size for at least 6000 years, it is possible that the mutations in the two families are identical by descent. If there were substantial fluctuations in the size of the Bedouin population, it is more likely that there were two independent mutations. Based on the available data, the population genetic analysis does not strongly favor one conclusion over the other.

Keywords

primary ciliary dyskinesia; Bedouin; founder mutation

INTRODUCTION

Primary ciliary dyskinesia (PCD, MIM 242650) refers to a heterogeneous group of genetic ciliopathies characterized by ultrastructural defects in the axoneme, the microtubule-based core of ‘9+2’ motile cilia, and sperm flagella (Afzelius , 1976; Bush et al., 2007). The incidence is estimated at 1:15,000-1:30,000 (Noone et al., 2004; Bush et al., 2007), with higher incidence in certain consanguineous and isolated populations (Jeganathan et al., 2004; Kennedy et al., 2007; Chilvers et al., 2003). Eight PCD-causing disease genes have been reported and these account for an estimated 17-38% of cases (Zariwala et al., 2006;

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Hornef et al., 2006; Failly et al., 2008; Failly et al., 2009). With the exception of the *KTU* gene, these genes all encode structural components of the force-generating axonemal outer dynein arm that is responsible for ciliary beat generation: *DNAH5* (Olbrich et al, 2002) and *DNAH11* (Bartoloni et al., 2002) (heavy chain dyneins); *DNAI1* (Pennarun et al., 1999) and *DNAI2* (Loges et al., 2008) (intermediate chain dyneins); *TXNDC3* (Duriez et al., 2007) (light chain dynein); *RSPH4A* and *RSPH9* (Castleman et al., 2009) (radial spoke head proteins). *KTU* encodes a cytoplasmic protein required for preassembly of the dynein arm, prior to its transport into the axoneme (Omran et al., 2008). No work has been published on common ancestors of the mutations identified in these genes.

Different ciliary ultrastructural defects have been described, each of which results in ineffective ciliary function. The most common are lack of inner and /or outer dynein arms, and more rarely ciliary disorientation, ciliary transposition or defective radial spokes (Afzelius et al., 2001; Chilvers et al., 2003). Clinical features include chronic respiratory infections leading to lung damage and sub fertility. About half of patients manifest laterality defects due to a randomization of left-right body axis determination, proposed to result from defective function of embryonic ‘9+0’ ultrastructure nodal cilia (Nonaka et al., 1998; Stannard et al., 2004; Kennedy 2007; Fliegauf et al., 2007). PCD heterozygotes have normal ciliary function and no clinical features of this disease.

A common mutation in the radial spoke head protein-encoding gene *RSPH9* was previously identified in two Bedouin families, one from Israel and one from the United Arab Emirates (UAE) (Castleman et al., 2009). Affected individuals in both families were homozygous for the *RSPH9* mutation c.801_803delGAA (p.Lys268del) that gives rise to cilia dysmotility associated with central-microtubular-pair abnormalities. We examined here whether the mutation arose in one distant ancestor in the families’ history and subsequently spread into two families, or whether it occurred independently in each of the two families. Herein we focused on the extended 5 generation Israeli Bedouin family, in which we analyzed mutation segregation, and calculated the age of the mutated allele in both families based on haplotypes and haplotype+microsatellite in an attempt to define the source of the mutation.

METHODS

Subjects

Two Bedouin families were studied, one from Israel and one from the UAE. Both were partially described in Castleman et al. (2009) and correlate to UCL152 and UCL146, respectively. The UAE family is detailed in Stannard, et al. (2004); the extended Israeli Bedouin family is detailed here.

Haplotype analysis

Haplotypes were constructed from microsatellite and single nucleotide polymorphism (SNP) genetic marker information using Haplopainter (Thiele et al., 2005). The genotyping data was generated as described previously (Castleman et al., 2009) and presented here. A merged marker map was created using the Illumina Linkage IVb SNP and deCODE Genetics microsatellite maps, and in-house microsatellites were positioned with reference to the University of California Santa Cruz genome browser (NCBI Build 36.1).

Mutation screening by restriction fragment length analysis

In this study, DNA was prepared using the salting out method (Miller & Polesky , 1988) from blood samples obtained from 18 members of the family. Each individual completed an informed consent form.

MboII restriction digestion was used to detect the c.801_803delGAA mutation as described previously (Castleman et al., 2009). Briefly, DNA samples were PCR amplified using primers 5′-CCAGTGGAACCATAGCACCT and 5′-AACAGGCAGGCCAAGTTCAC-3′ and PCR conditions of 5 mins at 94°C, 30 cycles of 1 min each at 94/ 62 /72°C, and a final 10 mins at 72°C, using a prepared 2xReddyMix PCR master mix (1.5mM $MgCl_2$) (Thermo Scientific). PCR products were cleaved by *MboII* yielding two fragments of 260 and 96 bp for normal alleles and a single fragment of 356 bp for mutant alleles.

Fifty healthy controls of Israeli Arab origin were screened for the mutation. The research protocol was reviewed and approved by the Israeli National Ethics Committee

Calculation of allele age

The time since different copies of an allele are descended from a common ancestor can be estimated from the length of the haplotype shared by all copies. The basic idea is that recombination steadily erodes the ancestral haplotype in each allelic lineage at a known rate. If initially the ancestral haplotype on one side of an allele is of length i (measured in nucleotides), then t generations later the probability that it is of length j

$$\begin{aligned}\Pr(j=i) &= (1-c)^{it} \\ \Pr(j<i) &= (1-c)^{jt} - (1-c)^{(j+1)t}\end{aligned} \tag{1}$$

is where c is the recombination rate per nucleotide (Slatkin, 2008). If the rate per nucleotide varies, then the distance is measured in map units instead of nucleotides.

From equation (1), the distribution of lengths of a haplotype shared by two independent lineages can be calculated. If the length of the ancestral haplotype in one lineage is of length j and in the other of length k, then the shared haplotype will be the smaller of j and k. The standard theory of order statistics Kendall and Stuart, 1977) states that the distribution of $l=\min(j,k)$ is

$$\Pr(l) = \left[(1-c)^{lt} - (1-c)^{(l+1)t}\right]^2 + 2(1-c)^{(l+1)t}\left[(1-c)^{lt} - (1-c)^{(l+1)t}\right]. \tag{2}$$

The first term represents the probability that both ancestral haplotypes are of length l and the second represents the probability that one is longer.

The data for a given pair of chromosomes is the pair of lengths of shared haplotypes on either side of the mutation, l_1 and l_2. Because the distributions of those lengths are independent, the probability of observing the data given t is

$$L(t|l_1, l_2) = \Pr(l_1 l_2|t) = \Pr(l_1|t)\Pr(l_2|t), \tag{3}$$

which is the likelihood of t as a function of the data. We can then estimate the age by finding the value of t that maximizes this likelihood. The curves in Fig. 3 labeled “Haplotype only” are graphs of Equation (3).

Further information about age comes from the fact that none of the microsatellites within the conserved haplotype in each family have mutated. We can account for the observation that no mutations at several microsatellite loci are detected in the data. We can incorporate that information into the likelihood by computing the probability of observing no mutations during the time since the two haplotypes were descended from a common ancestor. The

probability of no mutations in two lineages in t generations is $\exp(-2\mu nt)$, where n is the number of loci (20 or 11) and μ is the mutation rate per locus. Weber and Wong (1993) estimated μ to be 1.2×10^{-3}. Multiplying this exponential by the likelihood computed from the length of the shared haplotype gives a new likelihood, shown in Fig. 3 as Haplotype +microsatellite.

RESULTS

Two Bedouin families were previously analyzed, one from Israel and one from the UAE, that link to the *RSPH9* locus on chromosome 6p21.1 and carry the *RSPH9* c. 801_803delGAA mutation (Castleman et al., 2009). The Israeli Bedouin pedigree presented here in detail consisted of 5 generations (Fig. 1; Table 1): 83.3% of affected individuals had neonatal respiratory distress and /or ear disease and/or sinus disease. Respiratory infections in this family were due to *Sreptococcus pneumomia* (66.6%) and *Hemophilus influenzae* (50%). Lung functions deteriorated with age, from mildly impaired in infancy and childhood to severely impaired in all 5 adults, who had bronchiectatic changes, particularly in the lung bases, with one of them listed for lung transplant. Infertility or hypo fertility necessitated *in vitro* fertilization in 100% of affected adults. Patient IV-11 had died due to septic shock complications arising from immunosuppressive therapy for B-Cell lymphoma of the adrenal. Nasal brush biopsies in 2 patients were reported as normal or suboptimal and the ciliary beat frequency was not assessed. Clinical characterization of the UAE Bedouin family was described previously (Stannard et al., 2004).

Haplotypes in the two Bedouin families across the *RSPH9* locus on chromosome 6p21.1, based on genotyping data from the Castleman et al. (2009), are presented in Fig. 2. This defines a 10.4 Mb region of homozygosity identical-by-descent (IBD) in the UAE Bedouin family between *D6S291* and *D6S1638*, which overlaps with a smaller 1.9 Mb region in the Israeli Bedouin family between *D6S400* and *rs3734693*. Thus, in the two Bedouin families there is only a small shared 1.9 Mb of homozygosity that is IBD. Across this *D6S400-rs3736493* critical region, alleles are shared at only two in-house microsatellite markers, *UAEPal5* and *UAEPal3*, located on either side of *RSPH9*. These are present at 66.5 and 66.6 centiMorgan (cM) from the telomere of the p arm of chromosome 6 (Fig. 1). *RFLP* performed in the current study showed mutation segregation within the Israeli Bedouin family (shown in part in Fig. 1).

We used the haplotype information to investigate the history of this mutation in the Bedouin families according to the formulae presented in the Method section. Analyzing each family separately, we assumed that all copies of the mutation are identical by descent within each family and that the source of the mutation in each family was an individual t generations in the past. In the UAE Bedouin family, the shared haplotype extends from 56.86 to 70.14 cM, and there is no heterozygosity of any locus within that range. The mutation is at map position 66.55, and hence l_1=70.14–66.55=3.59 and l_2=66.55–56.86=9.69, where all distances are measured in cM. We used Equations (2) and (3) to compute the likelihood of t for the UAE Bedouin family (Fig 3A, ‘Haplotype only’ curve). The maximum likelihood estimate (MLE) of t is 7 generations. The support interval, within which the log-likelihood is less than 2 units from the maximum, is (1, 36). Incorporating the information about the lack of mutation in the microsatellite loci in the conserved haplotype (‘Haplotype +microsatellite’), the MLE of t is reduced to 5 generations and the support interval is (1, 17).

The shared haplotype is shorter for the Israeli Bedouin family, extending from 64.36 to 66.90 (l_1=66.90–66.55=0.45 and l_2=66.55–64.36=2.19). The likelihood curve is shown in Fig. 3B (‘Haplotype only’). The MLE of t based on the shared haplotype alone is i38 generations with a support interval of (6, 119). Within that shared haplotype, 11

microsatellite loci have not mutated. The log-likelihood curve that accounts for these microsatellites is also shown in Fig. 3B ('Haplotype+microsatellite'). The MLE of *t* is 30 and the support interval is (5, 95).

The mutation was not found in any of the 100 chromosomes screened from matched Israeli Arabs in this study

DISCUSSION

Bedouin are traditionally pastoral semi-nomadic Arab tribes that are spread out in North Africa and the Middle East. Historically, the Bedouin engaged primarily in nomadic herding, agriculture, raiding, and sometimes fishing. They also generated income by transporting goods and people across the desert. Scarcity of water and permanent pastoral land necessitated their constant movement. The Israeli Bedouin family described here is from a tribe that arrived from Libya at the end of the 19th century and settled near Gadera in the south of Israel. With the establishment of the State of Israel in 1948, they fled to Hebron, and several years later returned to Ramleh, a city in Israel where they were settled in housing provided by the government (Farag & Teebi, 1977; Kressel, 1974). This community is unaware of the other PCD family from the UAE and no historical connections could be gleaned from the known family history.

According to the genetic analysis in this study that demonstrated the mutation segregation among the individuals within the Israeli Bedouin family (Fig. 1), the mutation was introduced 5 generations ago by individual I-1 or I-2, the ancestors of the family, and subsequently spread to their 3 sons in generation II. Inter-familial marriages between first degree cousins in generations III and IV led to a total of 6 affected individuals in generations IV and V, respectively. These patients are the oldest known to carry the RSPH9 defect and display the full spectrum of the disease (Table 1). Their signs relate predominantly to infertility and the pulmonary system, which, when compared with the patients related to the siblings of the UAE family – ages 8 months and 4 and 5 years – showed marked impairment compatible with aggravation of pulmonary functions throughout lifetime in these patients. Bacterial infections were caused by pathogens similar to those reported for other PCD patients (Santamaria et al., 2008), but exhaled nitric oxide (ENO) test was not predictive Horváth et al., 2003).

One of the 5 adults in the Israeli Bedouin family had B cell adrenal lymphoma. Malignancy has not been typically correlated with PCD, but different tumors have been reported in a few cases of Kartagener's syndrome (Verdejo et al., 2000; Yoshida et al., 1986; Barselo et al., 2008; Nukina et al., 1989).

A larger screen for RSPH9 mutations and homozygosity of the c.801_803delGAA RSPH9 defect may require further study in light of evidence supporting the role of cilia in cell cycle regulation and downstream signaling (Fliegauf et al., 2007; Gerdes et al., 2009). Clinical investigations of nasal brush biopsies could result in under-diagnosis of PCD patients with the central microtubule defect associated with the c.801_803delGAA mutation, as occurred with 2 samples derived from the Israeli Bedouin family (Castleman et al., 2009), and consistent with the UAE Bedouin samples that demonstrated absent central microtubules in only 12.5% to 17% of the cilia on cross-sectional analysis (Stannard et al., 2004). The ultrastructural change involves an intermittent absence of the central microtubular pair, which could remain undetected since only a certain percentage of each epithelial cilia section would show the defect, leading to the detection of the defect in the remainder of the cilia above the plane examined (Stannard et al., 2004). Thus, a nasal biopsy from patients with this specific defect should be interpreted with extra caution in the context of this pitfall.

Although rare diseases are more common in inbred families, we were intrigued that two families of Bedouin origin harbor an identical rare mutation. Given that the incidence of PCD, which is a heterogenic disorder, is estimated at 1;15,000-1;30,000, the gene frequency defect should be 0.008-0.005, and the frequency of the c.801_803delGAA mutation markedly lower. Supporting this is the lack of this mutation in 200 Bedouin and Arab controls' chromosomes (Castleman et al., 2009), and an additional 100 chromosomes from matched Israeli Arabs analyzed in this study. These findings suggest that the mutation did not occur independently in each family.

If the mutations in the two families are descended from a common ancestor, we can use the same theory employed to estimate the age within each family to estimate the time since that common ancestor. In this case the shared haplotype is much shorter than within each family, only from 66.60 to 66.50. Therefore, $l_1=l_2=0.05$, and the likelihood of t for this case is shown in Fig. 4. The MLE is roughly 900 generations with support interval (150, 2900).

The support intervals for the estimate ages are broad, as is typical of estimates of allele age based on haplotype data. Although some studies claim much narrower confidence intervals for estimates of allele age, such narrow confidence intervals are based on simplifying assumptions that ignore one or more sources of uncertainty. (Slatkin and Rannala 2000) Although we cannot estimate the age of mutations within each family with great precision, we can conclude that the most recent common ancestor carrying the mutation in each family is recent, less than 17 generations ago in the UAE Bedouin family and less than 95 generations in the Israeli Bedouin family, probably much less in both families. Our estimates of age are conservative because the theory used assumes that the mutation is neutral. Any selection against heterozygous carriers would result in lower estimates of age both within and between families (Slatkin 2008). Our estimates are not affected by under-diagnosis because the method used does not assume every case is identified. We assume only that probability that an individual is diagnosed is not dependent on the length of the ancestral haplotype. In other words, we assume that the individuals analyzed represent a random sample of individuals with PCD. Of course, if genotypes of other individuals not included in our study because of under-diagnosis had ancestral haplotypes of different lengths, our analysis would obtain different estimates of allele ages.

We can also conclude that if the mutations in the two families are identical by descent, the original mutation is quite old, at least 150 generations, and probably much older. Whether such a large estimated age for a rare, deleterious mutation is plausible depends on the history of the population. In populations of constant size, low frequency alleles may well be old; the average age in this case is approximately (Kimura & Ohta, 1973) $\bar{t} = -4Nx \ln(x)/(1-x)$ (Kimura & Ohta, 1973); if $x=10^{-3}$ and $N=5000$, $\bar{t}=138$ generations. But, if the population has grown substantially in the recent past, the average age will be much younger (Slatkin 2002). With a minimum age of 150 generations and 25 years per generation, the population size would have to have been roughly at its current level for about 6000 years. Otherwise, it is less likely that the mutation in the two families is descended from a common ancestor and more likely that there were two independent mutations. Based on the currently available data, the population genetic analysis does not strongly favor one conclusion over the other.

The assessment strategy presented here to resolve allele age and origin may have implications for population genetics, public health considerations, and understanding the dynamics of mutation evolution in other diseases as well.

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Abbreviations

IBD	identical-by-descent
MLE	maximum likelihood estimate
PCD	primary ciliary dyskinesia
PCR	polymerase chain reaction
SNP	single nucleotide polymorphism
UAE	United Arab Emirates

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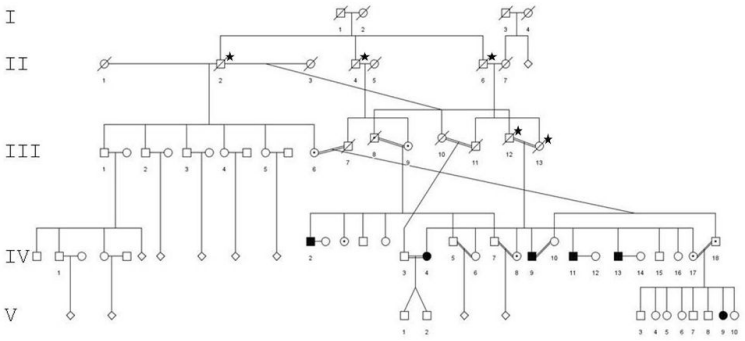

Figure 1.
Mutation segregation in the Israeli Bedouin family. Affected: filled circles and squares; dot within circle or square: carrier; asterisk: inferred carrier.

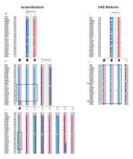

Figure 2.
High density haplotypes across the *RSPH9* locus on chromosome 6p21.1. SNP markers, indicated by the prefix 'rs', and microsatellite markers are shown against their genetic distance in cM from the p arm telomere of chromosome 6. Boxing indicates the regions of homozygosity across the *RSPH9* gene, with flanking markers for the *RSPH9* locus defined by loss of homozygosity in affected individuals. The *RSPH9* gene is located between markers *UAEPal3* and *UAEPal5*. Arrows indicate the Israeli Bedouin family critical region against that of UAE Bedouin family, which was not genotyped for as many SNP markers. Note that SNP information is inferred in family UCL152 for individuals III:12, V:3-8 and V: 10. No microsatellite data is inferred. Three healthy distant members in the Israeli family are not shown.

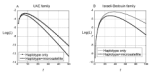

Figure 3.
Log-likelihood of the age of the common ancestor within the. UAE family (A) and the Israeli Bedouin family (B). The curves labeled ‘Haplotype only’ were computed from Equations (2) and (3) in the text. The curves labeled ‘Haplotype+microsatellite’ were computed by subtracting $-2n\mu t$ from the log-likelihood computed for haplotype only, where n=20 for the UAE Bedouin family and n=11 for the Israeli Bedouin family and $\mu=1.2\times10^{-3}$. The log-likelihoods were computed for integer values of t only.

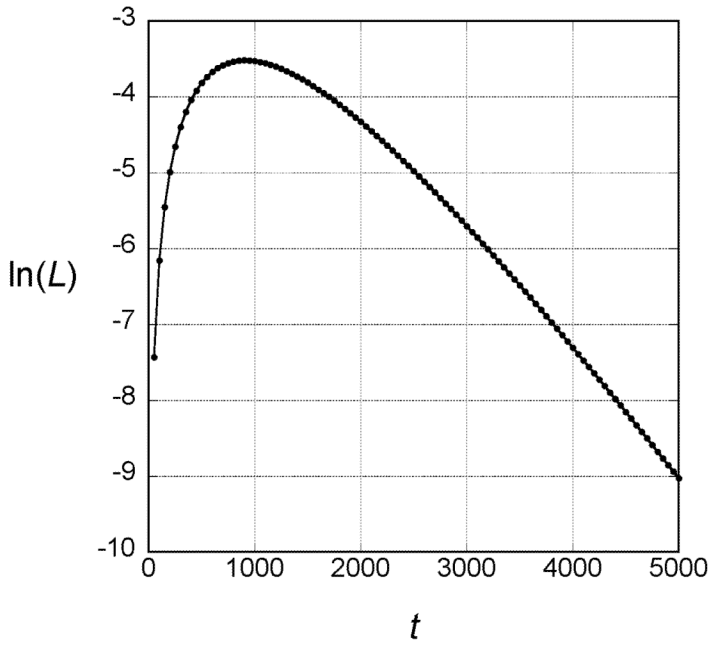

Figure 4.
Log-likelihood of the age of the mutant in the two families under the assumption that they are descended from a common ancestor. The results were obtained by evaluating Equations (2) and (3) in the text for *t* in multiples of 50 generations.

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Table 1

Clinical characteristics of affected individuals in the Israeli Bedouin family.

Affected individual	**IV-2**	**IV-4**	**IV-8**	**IV-11**	**IV-13**	**V-9**
Age (years)	45	43	50	42	48	9
Gender	M	F	M	M	M	F
Neonatal respiratory distress	−	+	+	+	+	+
Bilateral bronchiectasis	+	+	+	+	+	+
FEV1% predicted[a]	45	51	35.9	NA	63.8	70
Ear disease	+	AOM[b]	SOM[c]	+	AOM	−
Sinus disease	+	+	+	+	+	−
Infertility[d]	+	+	+	+	+	−
ENO[e] (ppb)	NA	NA	NA	NA	NA	7
EM[f]	NA	Normal	Normal	NA	NA	NA
Other			Lung transplantation pending	Died at 42 Y		

NA - not assessed

[a] FEV1 predicted - Volume Expired in 1st second, reflecting airflow obstruction degree: mild = 70-80%; moderate = 50-70%; severe <50%.

[b] acute otitis media

[c] serous otitis media

[d] females required IVF; males had abnormal sperm motility/azoospermia

[e] exhaled nitric oxide test - normal >2.4 ppb

[f] electron microscopy for nasal brush biopsy