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Report

The Paternal-Age Effect in Apert Syndrome Is Due, in Part, to the Increased Frequency of Mutations in Sperm

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A paternal-age effect and the exclusive paternal origin of mutations have been reported in Apert syndrome (AS). As the incidence of sporadic AS births increases exponentially with paternal age, we hypothesized that the frequency of AS mutations in sperm would also increase. To determine the frequency of two common *FGFR2* mutations in AS, we developed allele-specific peptide nucleic acid–PCR assays. Analyzing sperm DNA from 148 men, age 21–80 years, we showed that the number of sperm with mutations increased in the oldest age groups among men who did not have a child with AS. These older men were also more likely to have both mutations in their sperm. However, this age-related increase in mutation frequency was not sufficient to explain the AS-birth frequency. In contrast, the mutation frequency observed in men who were younger and had children with AS was significantly greater. In addition, our data suggest selection for sperm with specific mutations. Therefore, contributing factors to the paternal-age effect may include selection and a higher number of mutant sperm in a subset of men ascertained because they had a child with AS. No age-related increase in the frequency of these mutations was observed in leukocytes. Selection and/or quality-control mechanisms, including DNA repair and apoptosis, may contribute to the cell-type differences in mutation frequency.

Much has been written about the "mutagenic male" (Hurst and Ellegren 2002) and the higher male-to-female mutation rate in many genetic disorders (Vogel and Rathenberg 1975; Crow 2000). Conventional wisdom says that the greater number of germ-cell divisions in males compared with females contributes to the higher mutation frequency in males (Penrose 1955), which manifests as an increased incidence with paternal age of de novo cases of disorders, as well as paternally derived mutations (Moloney et al. 1996; Shuffenecker et al. 1997; Wilkin et al. 1998; Glaser et al. 2000). However, the linear increase with age in the number of divisions does not fully explain the exponential increase with paternal age in the incidence of affected children (Vogel and Rathenberg 1975; Risch et al. 1987; Crow 2000). The increased incidence with paternal age of affected children should reflect a corresponding increase with age in the frequency of mutations in sperm.

To test this hypothesis, we used Apert syndrome (AS) as a model. AS (MIM 101200) is characterized by craniosynostosis and syndactyly of the hands and feet and occurs in 1/65,000 to 1/160,000 live births (Blank 1960; Cohen et al. 1992). Of all sporadic cases, >99% are caused by one of two mutations in the fibroblast growth-factor receptor 2 (*FGFR2*) gene, 755C→G or 758C→G. The 755C→G mutation occurs in a CpG dinucleotide, whereas the 758C→G mutation does not. This difference may explain the higher mutation rate (5×10^{-6} vs. 2.7×10^{-6}) and incidence of children with AS (67% vs. 32%) with the former base-pair change (Park et al. 1995; Wilkie et al. 1995).

One hundred forty-eight men participated in this study from two independent study sites, which served as replicate samples. Fifty-seven were recruited from the greater

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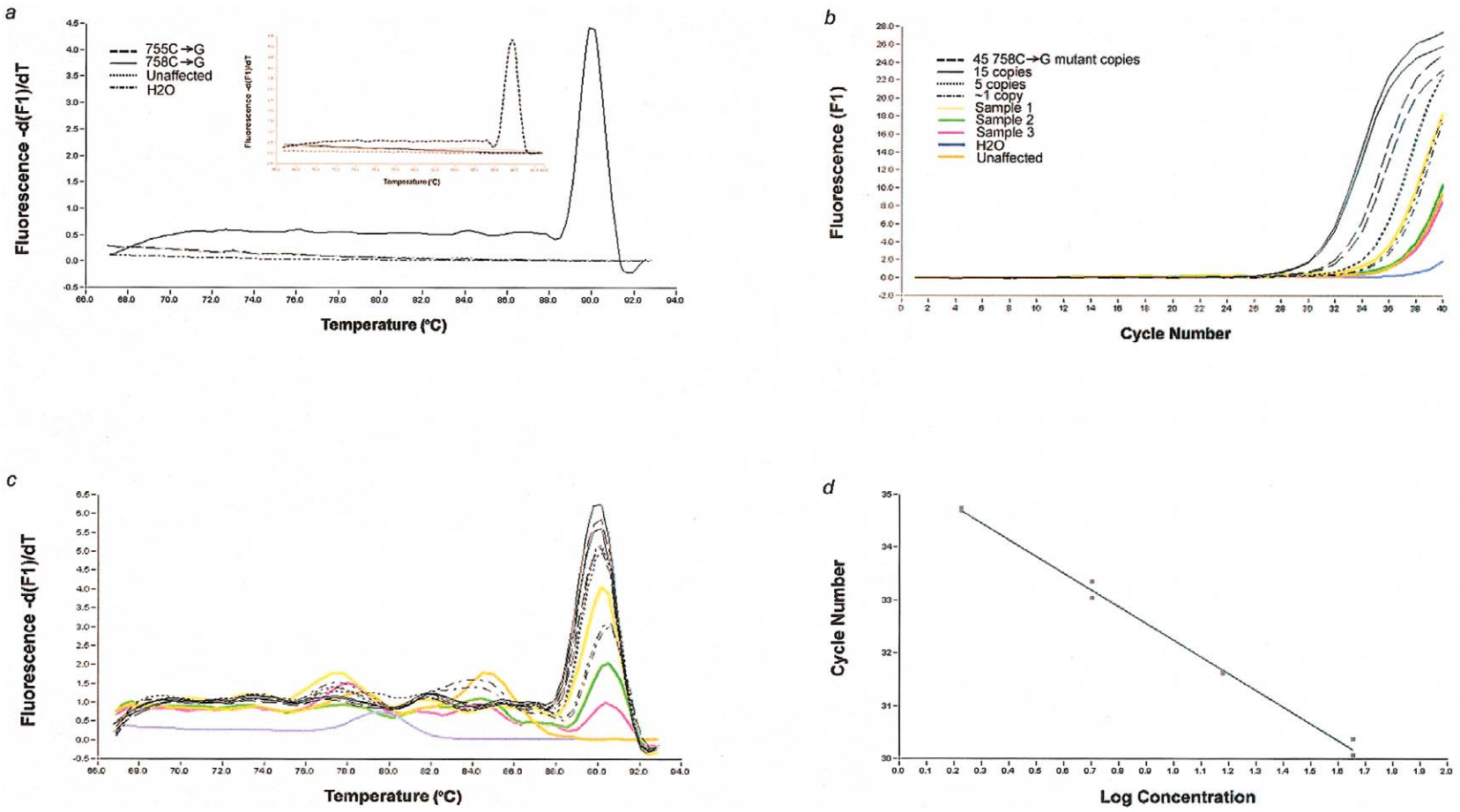

Figure 1 Specificity and sensitivity of the allele-specific PNA-PCRs. *a,* Melt-curve analysis after amplification of DNA from a patient with the 755C→G mutation yielded one specific peak. No cross reactivity with the other 758C→G mutation occurred. Inset shows melt-curve analysis for the 758C→G mutation with no cross reactivity for the 755C→G mutation. *b,* Amplification of standards and three samples of the 758C→G mutation. Standards used were 45, 15, 5, and 1 mutant copies diluted in human genomic DNA. Duplicates of each standard are shown. Negative controls are water and human genomic DNA. *c,* Melt-curve analysis after amplification of standards and samples from *b*. *d,* Standard curve for the 755C→G mutation with duplicates of each standard.

Baltimore area (Johns Hopkins Hospital [JHH]), (mean age 42.0 years, range 21–78 years), and 76 were acquired from the Lawrence Livermore National Laboratories (LLNL) (mean age 43.0 years, range 22–80 years) in Livermore, CA, as part of the University of California at Berkeley and the LLNL Age and Genetic Effects on Sperm (AGES) study (Eskenazi et al. 2003). These 133 men did not have children with AS (referred to herein as "controls"). Each of 15 unaffected men who had a child with AS was recruited from online support groups or through his child's physician in the United States (mean age 44.1 years, range 34–64 years). There were no statistical differences by two-tailed Student's *t* tests between the average ages of the JHH and AGES controls ($P > .6$) and between all 133 controls and the 15 fathers ($P > .6$). Of the 15 men who had children with AS, 6 had a child with the 755C→G mutation, whereas 1 had a child with the 758C→G mutation. None of the other eight men provided information about his child's mutation. Semen samples from JHH participants were received within a 24-h period and immediately aliquoted and stored frozen until DNA was isolated. Second semen samples, obtained at least 3 mo after the first samples, were provided by 35/57 JHH controls and 2/15 fathers. Blood samples were also obtained from 21/57 JHH controls but from none of the fathers of children with AS. Sample collection for the AGES study was similar, except that the samples were collected and processed within 2 h. Participants in both the JHH and AGES studies completed an epidemiological questionnaire. Subjects were excluded if they had a semen analysis with zero sperm count, a vasectomy, or a history of cancer; had received chemotherapy or radiation therapy for cancer; had smoked cigarettes in the last 6 mo; or had a fever >101°F in the last 3 mo (Eskenazi et al. 2003). Informed consent was obtained from men in both sample sets after approval was given by the appropriate institutional review board.

DNA was isolated from sperm and whole blood, according to PUREGENE protocols 00900 and 00050, respectively. To ensure the lysis and removal of all nonsperm cells, an additional incubation step, with Cell Lysis solution followed by centrifugation, was added. DNA isolations were performed one at a time in an isolated PCR hood that was UV irradiated for at least 20 min between samples.

The two recurrent mutations in AS, 755C→G and 758C→G, were detected using one-step allele-specific PCR assays. The specificity of these assays depended on a peptide nucleic acid (PNA) that masked the wild-type allele and allowed amplification from only the mutant allele (Orum et al. 1993). All PNA-PCR reactions were performed on the Roche LightCycler with the FastStart DNA Master SYBR Green I kit (Roche Molecular Biochemicals). Reactions were performed in a 20-μl volume with 150 ng DNA, equivalent to 50,000 sperm, and a final concentration of 0.5 μM of each primer, 2 μM PNA 5′-GATCGCCTCACCG-3′, and either 4 mM (for the 755C→G mutation) or 3.5 mM (for the 758C→G mutation) $MgCl_2$. Samples were analyzed 7–16 times, equivalent to 350,000–800,000 sperm, for each mutation. This number of trials allowed for a >90% chance of detection of one mutant sperm, assuming the incidence of mutant sperm would be similar to the birth incidence of AS. The reproducibility of the assays was ascertained by determining concordance between duplicate semen samples and between the JHH and AGES samples sets. Little evidence for a difference in either comparison was found ($P = .09$ and $P = .085$, respectively).

For the 755C→G mutation, primers 5′-TAGATTCAGAAAGTCCTCACC-3′ and 5′-CCTCTCTCCACCAGAGCGTTG-3′ were used to amplify a 224-bp product. PCR cycle conditions were: 10 min at 94°C, followed by 45 cycles of 15 s at 94°C, 5 s at 70°C, 5 s at 69°C, and 12 s at 72°C. For the 758C→G mutation, primers 5′-TAGATTCAGAAAGTCCTCACC-3′ and 5′-TCCACCAGAGCGATCGCG-3′ were used to amplify a 218-bp product. PCR cycle conditions were: 10 min at 94°C, followed by 40 cycles of 15 s at 94°C, 5 s at 70°C, 5 s at 66°C, and 12 s at 72°C. The primers that amplified the 755C→G mutation did not amplify the 758C→G mutations and vice versa (fig. 1*a*). Melt-curve analysis was performed immediately after amplification in every experiment to determine the presence of the specific product. Samples were analyzed by gel electrophoresis to confirm that the single peak observed in the melt-curve analysis corresponded to a band of the correct size (data not shown). PCR products were then sequenced to confirm amplification from only the mutant allele (data not shown).

Standard curves were constructed for the 755C→G and the 758C→G mutations by diluting genomic DNA from an affected patient heterozygous for each mutation with human genomic DNA (Roche Molecular Biochemicals). The standards used in each experiment were 45, 15, 5, and 1 mutant copies in human genomic DNA. The number of mutant sperm per 50,000 was determined from the standard curve. If the cycle threshold of a sample was greater than that of the last standard and the melt-curve analysis showed the presence of mutant sperm, we assigned a value of 1 mutant sperm in 50,000 to this sample, the lowest possible biological value in our assay (fig. 1*b* and 1*c*). One copy of the mutation in a background of 50,000 wild-type copies, or haploid sperm, repeatedly amplified and showed a specific peak on the melt-curve analysis, whereas negative controls, wild-type human genomic DNA, and water did not (fig. 1*b–d*). The assays were not accurate with fewer than 1/50,000 mutant sperm.

Among 133 men who did not have a child with AS, the frequency of sperm with either mutation increased with age (fig. 2*a* and 2*b*). To assess the statistical signifi-

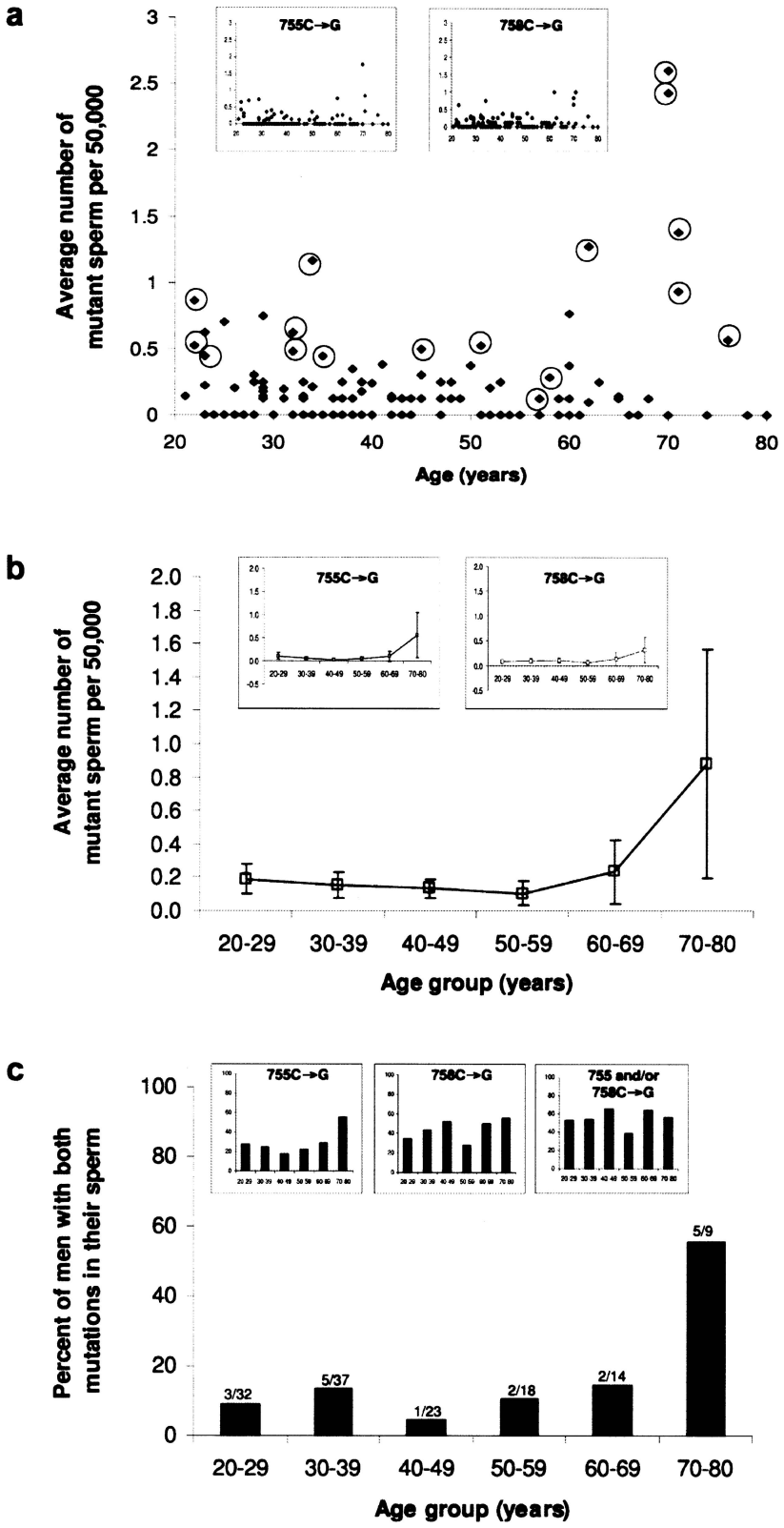

Figure 2 Mutation frequency with age. The number of sperm with either *FGFR2* mutation increases with age. *a*, Average for a given individual. A single diamond symbol may represent more than one individual with the same age and mutation frequency. *b*, Average for a given decade. Error bars indicate the 95% CIs. *c*, Percent of men in each age group with both mutations in sperm increases with age. Above each bar is the number of men with both sperm mutations over the total number of men in each age group. Insets are for each mutation or either mutation, as noted. Circles in panel *a* indicate those individuals who had both mutations in their sperm.

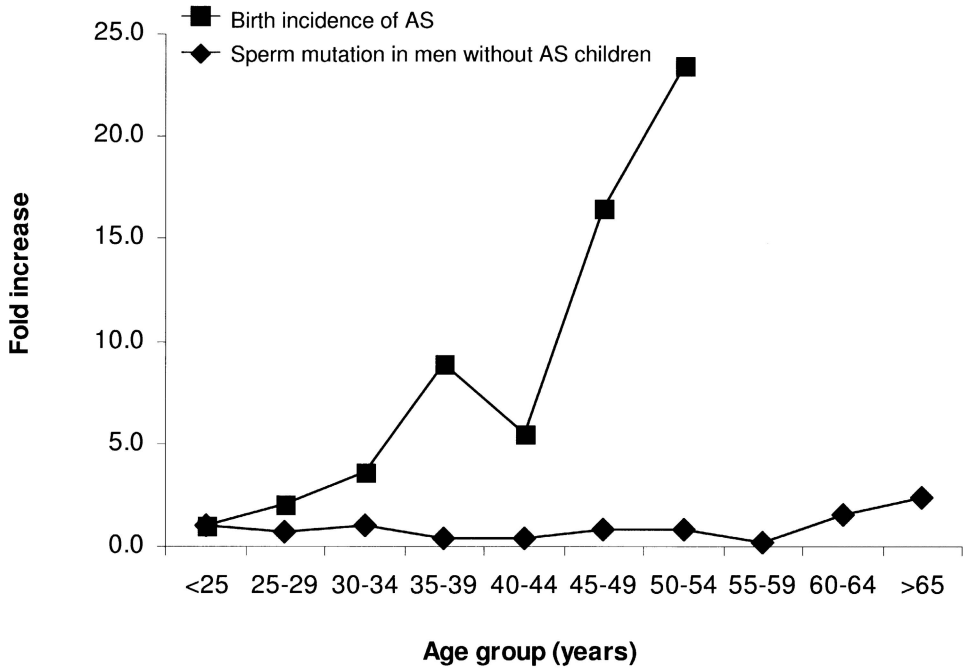

Figure 3 Comparison between the sperm mutation frequency (data from fig. 2*a*) and frequency of birth of children with AS (Risch et al. 1987), by use of fold change with respect to the value for age <25 years.

cance of this observation, we used a generalized linear model (McCullagh and Nelder 1989), with the assumption that the number of mutant copies in a sperm sample was Poisson distributed with mean increasing exponentially with age, and we performed a permutation test (Manly 1997) with 1,000 permutation replicates. All statistical analyses were performed with the software R, version 1.7.0 (Ihaka and Gentleman 1996). This analysis showed significant evidence for an increase with age in mutation frequency in these control individuals ($P = .008$). An age-related increase in the mutation frequency was also observed when the two individual mutations were considered separately ($P = .025$ for 755C→G; $P = .008$ for 758C→G). This increase, along with an increase in variability in mutation frequencies among individuals in a given decade, was greatest in men aged >60 years (fig. 2*b*). Sperm from men aged >70 years were more likely to have both the 755C→G and 758C→G mutations (20–29 years vs. 70–80 years; $P = .013$; Fisher's exact test; fig. 2*a* and 2*c*), suggesting that DNA-repair mechanisms may decrease in efficiency with age. In support of this hypothesis, recent studies have shown downregulation and decreased activity of several DNA-repair proteins in sperm from older mice and rats (Vani and Rao 1997; Intano et al. 2002).

Having demonstrated that the frequency of AS mutations in sperm increases in the oldest age groups, we wanted to compare these results with data on de novo cases of AS. To do so, we determined if the distribution of paternal ages for AS births (Risch et al. 1987) was similar to that which would be expected, given the observed mutation frequencies in sperm. We considered the seven age groups 19–24, 25–29, 30–34, 35–39, 40–44, 45–49, and 50–59 years and assumed that the mutation frequency in a sperm sample for a subject in an age group A was Poisson distributed with mean λ_A. The data on paternal ages from Risch et al. (1987) were assumed to follow a multinomial distribution, with q_A denoting the probability that the father of a child with AS is in age group A. Let p_A denote the proportion of fathers who are in age group A, taken from census data cited in Risch et al. (1987) and assumed to be known without error. By Bayes's rule, the age-specific mutation frequencies in sperm, λ_A, imply that the expected proportion of fathers of children with AS who are in age group A would be $\lambda_A p_A / \Sigma \lambda_A p_A$. We tested the hypothesis that, for all age groups, $q_A = \lambda_A p_A / \Sigma \lambda_A p_A$, by a likelihood-ratio test. Statistical significance was assessed via a parametric bootstrap, with 1,000 bootstrap replicates. We found that the increased frequency of mutant sperm in the oldest age groups is in contrast with the exponential increase in AS-birth frequency, which begins at age ~37 years ($P = .001$; fig. 3) (Risch et al. 1987). These results are consistent with those from a previous study of sporadic achon-

droplasia (MIM 100800), which show a discrepancy between the frequency of the *FGFR3* 1138G→A mutation in sperm and at birth (Tiemann-Boege et al. 2002). This indicates that the paternal-age effects in both AS and achondroplasia are not entirely due to the increased frequency with age of mutations in sperm.

The above data were based on men in the general population who had not fathered a child with AS and excluded any men who had a child with AS. To test the hypothesis that these groups of men may differ in their mutation frequencies, sperm DNA from men who had children with AS were analyzed for both *FGFR2* mutations. We found that these 15 men had significantly greater mutation frequencies at an earlier age, compared with men who had fathered unaffected children (n = 69; P = .002) or to all men, whether or not they had children (n = 133; P = .001)(fig. 4). These P values were obtained by a permutation test using the generalized linear model described above, with group-specific slopes and intercepts. The observed mutation frequency in sperm from men who had children with AS appears to approximate the birth frequency of AS more closely than the mutation frequency from control men. Of the 10 men aged <45 years who had children with AS, 3 had mutation frequencies (~1/50,000 mutant sperm) that were similar to those in 5/23 60–80-year-old control men. This percentage (30%; 3/10) of fathers of children with AS is significantly different, compared with the percentage of control men (2.4%, 2/85) age <45 years who had similar mutation frequencies (P = .008, Fisher's exact test).

The overall estimated mutation frequency, averaged across all ages, was 11 per 1 million sperm (95% CI 3–19 per 1 million) in fathers who had a child with AS and 3.9 per 1 million sperm (95% CI 2.9–5.2 per 1 million) in men who did not have a child with AS, consistent with the former group having a higher occurrence of mutant *FGFR2* sperm or level of mosaicism. This higher level of mosaicism among younger men who had fathered a child with AS has implications for genetic counseling, as it would increase their risk of having an-

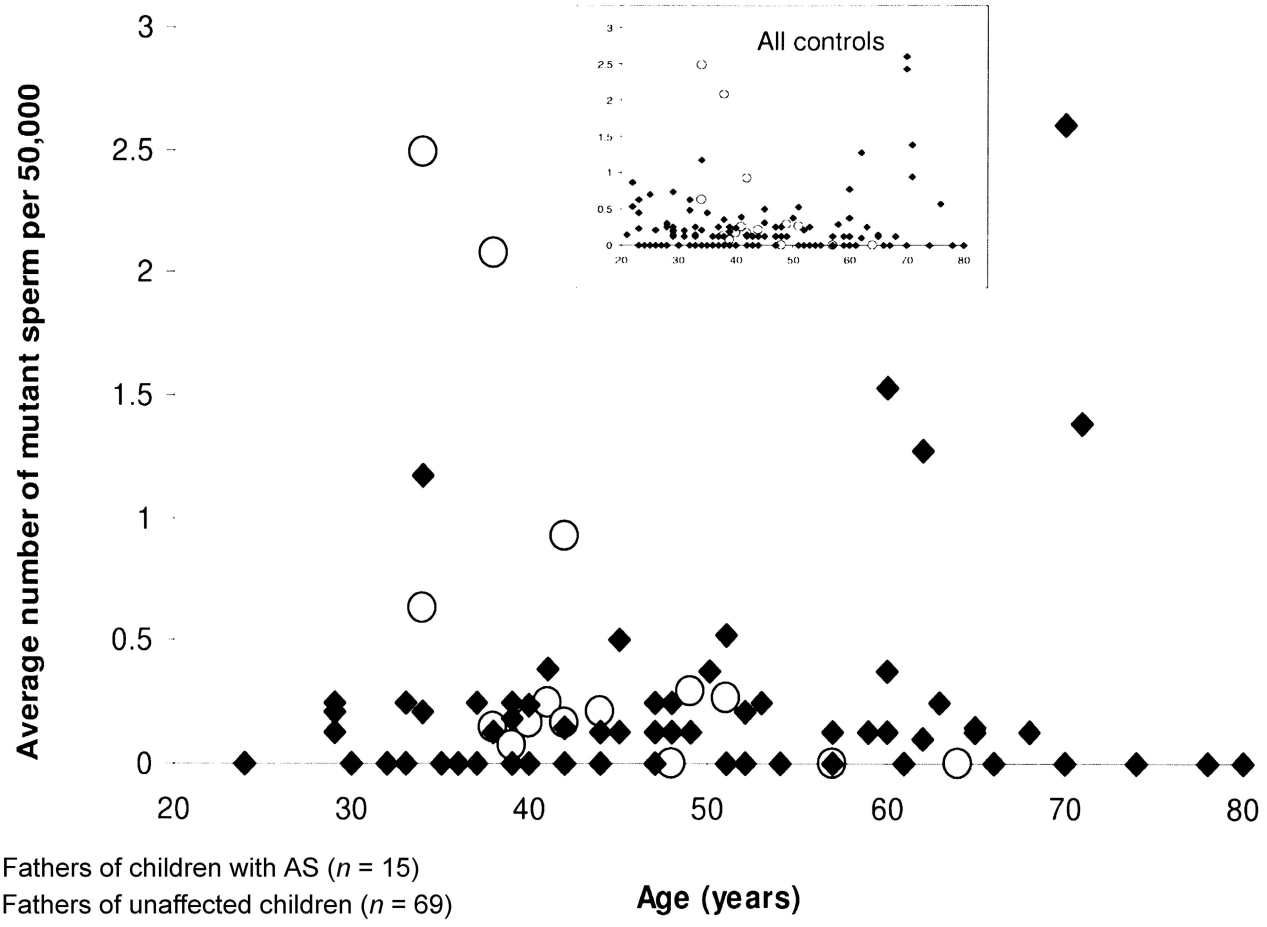

Figure 4 Comparison of the mutation frequencies in men who have and have not fathered a child with AS. The inset shows the comparison between men with a child with AS and all men, whether or not they had fathered a child.

other affected child. This observation could result from bias of ascertainment or earlier mutational events, possibly owing to an increased susceptibility to mutations, in fathers of a child with AS. Although we excluded anyone who had obvious exposure to mutagens, such as radiation therapy or chemotherapy, there may be other more subtle environmental factors that we are unaware of at this time that could influence mutation rate. It is possible that the risk of fathering a child with AS may increase even more as the men become older, because these mutational events, presumably occurring in the spermatogonial stem cells, would accumulate with age. However, we could not determine how the mutation frequency increased with age among men who have children with AS, as there were only three fathers aged >50 years in this group.

To test the hypothesis that the increase with age in mutation frequency in sperm corresponds to an increase in somatic cells, we analyzed for both mutations in blood samples from men in this study. No increase with age in mutation frequency, in particular in men aged >60 years, was observed in white blood cells ($P = .71$), a result that is significantly different from that observed in sperm ($P = .001$; paired permutation test) (fig. 5). AS mutations may not accumulate with age in white blood cells as they do in sperm, even though both arise from constantly regenerating stem cells, because of cell-type specific selection (Murdock et al. 2000).

Differences between the two mutations may also play a role in selection. These gain-of-function mutations, although occurring in adjacent amino acids, result in differing ligand affinities and specificities of the mutant tyrosine-kinase receptors (Yu et al. 2000; Ibrahimi et al. 2001). Although the 755C→G mutation accounts for twice as many cases of AS as does the 758C→G mutation, each is present at approximately the same frequency in sperm (1/500,000), which suggests either that the 755C→G mutation confers a selective advantage to sperm or that the 758C→G mutation is selected against in sperm or in utero. The 755C→G mutation, which occurs in a CpG dinucleotide, is notable because C→T or G→A transitions tend to occur more often than transversions in this context. The fact that the less-common transversion is observed more often at this base suggests selection for this specific nucleotide change.

In conclusion, our study suggests that there are many contributing factors to the paternal-age effect in AS. It cannot be explained solely by the increase in mutation frequency in sperm, which occurs much later in life and is not as exponential as the birth frequency. We found that fathers of children with AS are a subgroup of men in whom the increased frequency of mutant sperm occurs

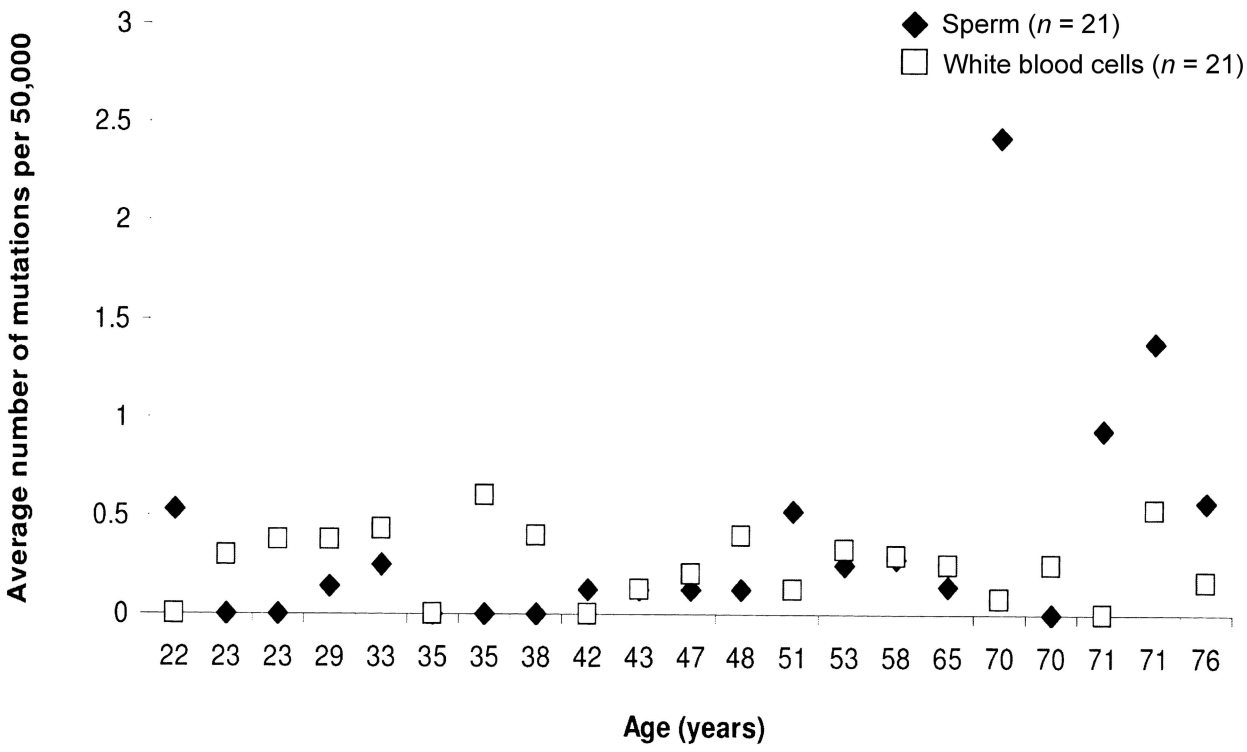

Figure 5 Comparison of the mutation frequencies in sperm and white blood cells from the same individual in a subset of 21 men who did not have a child with AS.

at an earlier age, which may contribute to the paternal-age effect. Selection for sperm with mutations may be another contributing factor, as suggested by our analysis of two different mutations. These findings may extend to other disorders with paternal-age effects, such as achondroplasia, Crouzon (MIM 123500) and Pfeiffer (MIM 101600) syndromes, and multiple endocrine neoplasia type 2A (MEN2A [MIM 171400]), but further study is needed. We also show that increases with age in mutation frequency and number of different mutations are not observed in white blood cells, which suggests that age effects are cell-type specific. Accumulated environmental exposure, reduced efficiency of DNA-repair mechanisms or apoptosis, or altered physiological selection during spermatogenesis may be involved in the differences observed between mutation frequencies. In-depth genetic and environmental studies may help to delineate subgroups of men at higher risk for having affected children.

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Electronic-Database Information

The URL for data presented herein is as follows:

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for achondroplasia; Apert, Crouzon, and Pfeiffer syndromes; and MEN2A)

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