

Association Analysis Between the Tag SNP for Sonic Hedgehog rs9333613 Polymorphism and Male Sexual Orientation

BINBIN WANG,*†|| SIRUI ZHOU,†‡|| FUCHANG HONG,* JING WANG,†‡ XIAOLI LIU,* YUMAO CAI,* FENG WANG,* TIEJIAN FENG,* AND XU MA†‡§

From the *Shenzhen Center for Chronic Disease Control and Prevention, Shenzhen, China; the †National Research Institute for Family Planning, Beijing, China; the ‡Graduate School, Peking Union Medical College, Beijing, China; and the §World Health Organization Collaborating Centre for Research in Human Reproduction, Beijing, China.

ABSTRACT: Male sexual orientation has been proposed to have genetic components, but previously suggested candidate genes have all received negative results. The human sonic hedgehog (*SHH*) gene is located in the 7q36 region, which was linked to male sexual orientation in a previous genome-wide association study. *SHH* is known to play an important role in embryo patterning, and there is evidence connecting it to sexual orientation. In this study, we performed an association analysis of the *SHH* tag single nucleotide

polymorphism rs9333613 in 361 subjects and 319 Chinese male controls. We find a significant difference in genotype and allele distribution between identified homosexuals and heterosexual control subjects, suggesting that the *SHH* gene could potentially be associated with male sexual orientation.

Key words: Androgen, fertilization, hormone, infertility, reproductive genetics, homosexuality, embryo patterning, genotyping.

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Homosexuality refers to romantic or sexual attraction or behavior among members of the same sex, either situationally or in an enduring disposition (American Psychological Association, 2007). Homosexuals constitute approximately 2%–6% of the male population (Laumann et al, 1994; Wellings et al, 1994), and research has shown evidence for a biological basis of sexual orientation. The most recognized evidence consists of neuroanatomical differences that have been reported for 3 brain regions in males: the arginine vasopressin neuronal population of the supra-chiasmatic nucleus (Swaab and Hofman, 1990; Zhou et al, 1995), the third interstitial nucleus of the anterior hypothalamus (INAH-3; LeVay, 1991), and the anterior commissure (Allen and Gorski, 1992). In all instances of significant differences, homosexual males appear to be neuroanatomically closer to females than average males.

Research on twin studies (Bailey and Pillard, 1991) showed that male sexual orientation might be at least partially heritable. This conclusion was supported by population-based studies using systematic ascertainment methods that reported heritability estimates of approximately 60% (Kendler et al, 2000). The genetic mechanism of human male sexual orientation has been debated among researchers; however, evidence of specific genes related to human male homosexuality has yet to be found.

The most compelling observation supporting a genetic component of homosexuality is a report linking human male homosexuality to microsatellite markers on X chromosome position Xq28 (Hamer et al, 1993; Hu et al, 1995). Other studies, however, containing different and larger sample sizes question this discovery (Rice et al, 1999). Candidate genes included in several studies, such as the androgen receptor gene (Macke et al, 1993) and aromatase cytochrome P450 (*CYP19*; DuPree et al, 2004) have also received negative results; so far, no genes have been successfully correlated with human male sexual orientation.

One genome-wide study of sexual orientation in males, however, showed promising results and revealed a high multilocus log odds ratio score in 7q36 with approximately the same maternal and paternal contributions. This suggests the possibility that genes in the 7q36 region might potentially correlate to male sexual orientation (Mustanski et al, 2005). The sonic hedgehog (*SHH*) gene, located in 7q36, is one of the family of

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Correspondence to: Prof Xu Ma, Center for Genetics, National Research Institute for Family Planning, 12, Dahuisi Rd, Haidian, Beijing, 100081 China (e-mail: nicgr@263.net); or Prof Tiejian Feng, Shenzhen Center for Chronic Disease Control and Prevention, No. 2021, Buxin Rd, Luohu District, Shenzhen, Guangdong, China (e-mail: tiejian.feng@gmail.com).

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|| These authors contributed equally to the work.

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genes related to the *Drosophila* gene hedgehog (*hh*) that encodes inductive signals during embryogenesis (Echelard et al, 1993; Roelink et al, 1994). Mammalian *Shh* encodes a signal that is instrumental in patterning the early embryo. It defines the pattern of neuronal cell types generated in the neural tube (Ericson et al, 1996) and controls patterns at a distance in brain development. *Shh* also affects the size, shape, and orientation of cell populations produced relative to the geometry of the morphogen source (Agarwala et al, 2001), the posterior of the limb buds (Riddle et al, 1993), and the anterior-posterior limb axis (Riddle et al, 1993). Malformations originating from a modification of SHH signaling include patterning defects of the autopod, preaxial syndactyly, and postaxial polydactyly (Gofflot et al, 2003). The *SHH* gene plays an essential role in mediating polarizing activity and inducing mirror image duplications (Riddle et al, 1993), hemisphere separation (Roessler et al, 1996), and left-to-right asymmetry (Tsukui et al, 1999). These roles of *SHH* could imply a link to homosexuality, especially given that various anthropometric studies, including metrics such as left-handedness and leftward dermatoglyphic asymmetry, have been linked to both sexual orientation and various growth factors (Hall and Kimura, 1994).

In this study, we examine the gene *SHH* in the 7q36 region and its function in development as related to expressive characteristics of homosexuality. Using HapMap, we investigated the tag single nucleotide polymorphism (tag SNP) rs9333613, which represents the gene *SHH*, and studied the relative occurrence of the polymorphism in homosexuals and control subjects to determine whether *SHH* is correlated with male sexual orientation.

Materials and Methods

Participants

A total of 361 Chinese male individuals identified as men who have sex with men (MSM) and 319 male controls (identified as heterosexuals) were recruited by Shenzhen chronic disease hospital, China. Shenzhen, being a city open to millions of tourists, tends to have a friendly attitude toward homosexual populations. The study subjects were recruited in 2 clinics. MSM subjects were recruited from clinics specializing in the treatment of homosexual males with either sexually transmitted diseases or acquired immunodeficiency syndrome. Healthy heterosexual control subjects were recruited after a medical examination. MSM individuals were selected according to the following criteria: self-identification as homosexual and having had contact (with another man/other men) at least once during the last 6 months. Controls were selected from an adult health check as self-identified heterosexuals that have never had same-sex contact. The ages of the participants were between 18

and 55 years. Participants having a history of drug use and mental disease, prostitution, or situational sexual behavior were excluded; informed consent was obtained from all participants. The informed consent given to the participants specified that a genetic resource database was going to be set up and that a wide range of "MSM candidate gene studies" were going to be performed. The study protocol was approved by the ethics committee of the National Research Institute for Family Planning, and written consent was obtained from all participants.

Genotyping

Blood samples from all MSM subjects and controls were collected and stored at -20°C . Genomic DNA was extracted from peripheral blood leukocytes with the use of QIAamp genomic DNA kits under the standard protocol provided by the manufacturer.

The rs9333613 polymorphism was proposed as the only tag SNP for the *SHH* gene by Haploview using Tagger pairwise with R² cutoff and MAF cutoff set at 0.8 and 0.1, respectively.

Genotyping the *SHH* gene rs9333613 polymorphism was performed by polymerase chain reaction (PCR) with the following primer set designed with Primer Premier 5.0: 5'-GCC TGA GGT CTC GGA GTC-3' and 5'-GGC CCT GGA TAG AAG CTG TC-3'.

The PCR consisted of a denaturing step at 94°C for 5 minutes; a 40-cycle amplification at 94°C for 30 seconds, 61°C for 40 seconds, and 72°C for 30 seconds; and a final extension at 72°C for 10 minutes. In the PCR controls, double-distilled water (ddH₂O; 2 μL each) was used as a replacement for the 2 μL of template.

The 442-bp PCR product was examined by agarose gel with ethidium bromide and then directly sequenced on an ABI 3730XL automated sequencer (Applied Biosystems, Foster City, California) to genotype each of the samples and controls.

Three different genotypes were defined for the individual polymorphisms: the homozygous wild type A/A, the heterozygous variant A/G, and the homozygous variant G/G.

Statistical Analysis

The allelic and genotypic distributions of the *SHH* rs9333613 polymorphism were estimated by allele counting, and the MSM and control groups were compared with the chi-square test.

Differences between genotype distribution and allele frequencies were tested by chi-square analysis. Statistical analyses were carried out with the Statistical Package for Social Sciences version 10.0 (SPSS 10.0, Chicago, Illinois). The criterion for significance was set at $P < .05$.

Results

Genotype and wild-type vs mutated allele frequencies were determined for MSM samples and controls.

The genotype and allele frequencies are summarized in the Table. We compared genotypes carrying at least

Table. Genotype distribution and relative allele frequencies of the rs9333613 polymorphism in the SHH gene in MSM subjects (n = 361) and controls (n = 319)

Group	No.	Genotype Frequency (%)			Allele Frequency (%)	
		A/A	A/G	G/G	A	G
MSM	361	230 (63.7)	121 (33.5)	10 (2.7)	581 (80.4)	141 (19.6)
Controls	319	237 (74.3)	78 (24.5)	4 (1.2)	552 (86.5)	86 (13.5)
		$\chi^2 = 9.410, df = 2, P = .009$			$\chi^2 = 8.914, df = 1, P = .03$	

Abbreviations: A/A, homozygous wild type; A/G, heterozygous variant; G/G, homozygous variant; MSM, men who have sex with men; SHH, sonic hedgehog.

1 mutation (A/G or G/G) with the wild type (A/A) (Figure). The distribution of genotype frequencies in MSM subjects and controls were in Hardy-Weinberg equilibrium. The results showed significant differences between 361 MSM males and 319 controls ($P < .05$) in all 3 rs9333613 genotypes, as well as both A and G alleles ($\chi^2 = 9.410, df = 2, P = .009$ with genotype; $\chi^2 = 8.914, df = 1, P = .03$ with allele), indicating that this polymorphism could be positively related to male sexual orientation.

Discussion

According to Mustanski et al (2005), a genome-wide study of male sexual orientation led to a certain position on chromosome 7: 7q36. This region deserves a denser mapping using tag SNPs as markers, but here, we examined 1 SNP in the gene *SHH*, which is the most promising candidate in this region for having a role in sexual orientation. Our association analysis reveals a promising result.

SHH-dependent proliferation of prospective digit progenitor cells is essential for specifying the complete pattern of digits across the anteroposterior axis (Towers et al, 2008). This left-to-right asymmetry in both brain and limb might indicate that the gene is related to the trait. One report showed that only homosexual men with 2 or more elder brothers had hyper-masculinized right-hand 2D:4D ratios (Williams et al, 2000), and

another proposed that homosexual men possess a more female-typical left-hand-finger dermatoglyphic pattern than heterosexual men. Another study compared the rates of non-right-handedness in homosexual and heterosexual participants and discovered homosexual participants had greater odds of being non-right-handed (Lalumiere et al, 2000).

Moreover, studies using *Drosophila* discovered several altered gene expressions related to homosexual behavior among male flies. Changes in the ecdysone receptor gene (*EcR*; Ganter et al, 2007), the fruitless gene (*Fru*; Kimura et al, 2005), and the *white* gene (Vett et al, 2002) all resulted in male-male sexual behavior, further suggesting that animal sexual orientation could have a genetic basis. Additionally, another study suggested that *SHH* signaling regulated expression of the *ABC* transporters. These transporters are mammalian homologs of the *Drosophila white* gene, and it has been shown that *white* overexpression might lead to homosexual male courtship in flies (Sims-Mourtada et al, 2007).

Additionally, the *SHH* signaling pathway, which has essential roles in developmental patterning in the early embryo, is required for the regeneration of prostate epithelium (Karhadkar et al, 2004) and signaling during induction of the hindgut region (Sasaki et al, 2004). All of the mentioned evidence suggests a link between *SHH* and homosexuality. This is in accord with our discovery that the tag SNP rs9333613 polymorphism, which represents the *SHH* gene, could be associated with male sexual

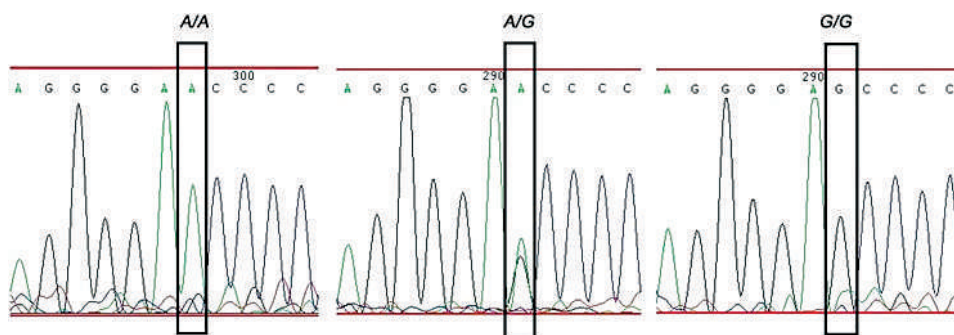


Figure. Three genotypes of the rs9333613 polymorphism in the sonic hedgehog gene. A/A indicates homozygous wild type; A/G, heterozygous variant; G/G, homozygous variant. Color figure available online at www.andrologyjournal.org.

orientation. We suspect that genotypes with at least 1 mutated allele in the rs9333613 polymorphism might have an effect on the expression of *SHH* and potentially be associated with male homosexuality along with many other undiscovered factors.

Because both genotype and allele frequency distribution differences between homosexual and heterosexual men reached significance, we suggest that the *SHH* gene could be a potential candidate gene associated with this behavior. However, this study still requires further investigation in different ethnic populations and more functional studies to discern the influence of the rs9333613 polymorphism on *SHH*.

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