Genetic variants in the *ETV5* gene in fertile and infertile men with nonobstructive azoospermia associated with Sertoli cell–only syndrome

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Objective: To assess the association between genetic variants in the *ETV5* gene with nonobstructive azoospermia (NOA) associated with Sertoli cell–only (SCO) syndrome.
Design: Genetic association study.
Setting: University.
Patient(s): Australian men (65 SCO, 53 NOA, and 242 fertile men) and American men (86 SCO and 54 fertile men).
Intervention(s): Paraffin-embedded human testicular tissue was sectioned and processed for immunofluorescence. Direct DNA sequencing and polymerase chain reaction–based SNP detection were performed to define genetic variants in the *ETV5* gene.
Main Outcome Measure(s): The localization of ETV5 in the human testis and the presence of *ETV5* genetic variants in fertile and infertile men.
Result(s): ETV5 is localized to the cytoplasm and nucleus of Sertoli and germ cells in adult human testes. We identified six previously reported and six new genetic variants in the *ETV5* gene. Of these, the allele frequency of the homozygous +48845 G>T (TT allele) variant was significantly higher in the SCO and NOA Australian men compared with fertile men.
Conclusion(s): The homozygous +48845 G>T (TT allele) variant confers a higher risk for male infertility associated with NOA and SCO in Australian men. (Fertil Steril® 2012;98:827–35. ©2012 by American Society for Reproductive Medicine.)
Key Words: ETV5, nonobstructive azoospermia, Sertoli cell–only syndrome, spermatogonia stem cells, male infertility

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Approximately 10% of couples worldwide struggle with fertility problems and at least one-third of these cases result from male factor infertility (1, 2). In recent years, there has been increasing evidence to suggest that genetic factors are an important cause of male infertility. Known genetic causes of male infertility comprise defects ranging from chromosomal anomalies to monogenic mutations. A range of numeric and structural chromosomal defects are found in ~7% of azoospermic and oligozoospermic men, most often Klinefelter syndrome (47,XXY) and microdeletions of the long arm of the Y chromosome, whereas monogenic causes of human

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infertility are rarely described but include mutations in the cystic fibrosis transmembrane conductance receptor, several genes associated with axoneme function and primary ciliary dyskinesia, aurora kinase C, phospholipase C zeta, and zona pellucida binding protein 1 (3–6). Several other genetic variations have been proposed as risk factors for male infertility (7–10).

Spermatogenesis is an intricate and dynamic process that transforms undifferentiated diploid spermatogonial stem cells (SSCs) into terminally differentiated spermatozoa within the seminiferous epithelium. Although the process begins in fetal life in mammals, male germ cells undergo mitotic arrest before regaining their proliferative and differentiative capacity at puberty, after which spermatogenesis continues throughout life. Divisions of SSCs create daughter cells that either maintain their stem cell identity or commit to differentiation into sperm. As such, a balance between SSC self-renewal and differentiation is critical for sustaining spermatogenesis and male fertility. The precise homeostasis of these processes requires intercellular communication between SSCs and their supporting somatic cells, particularly Sertoli cells, within the SSC niche (11). Dysregulation of either SSC self-renewal or differentiation can lead to the complete disruption of spermatogenesis and result in a phenotype referred to as "Sertoli cell–only" (SCO) syndrome.

SCO manifests clinically as a subset of nonobstructive azoospermia (NOA), a collective term for spermatogenic failure leading to absence of sperm in the ejaculate. SCO is characterized by the absence of all germ cell types on testicular biopsy and may follow defined insults (e.g., radio/chemotherapy, orchitis), but it is often idiopathic and likely of genetic origin (12). The only hope for biologic paternity in men with SCO is surgery to retrieve sperm from the rare pockets of spermatogenesis that may survive within the testes. Such testicular sperm extraction (TESE) may be performed by random biopsy or by microdissection TESE using an operative microscope, whereby recovery rates of 57% have been described in NOA generally (13) and 24% in NOA associated with pure SCO histology (14). Unfortunately, there are no noninvasive approaches to reliably predict the presence or absence of retrievable testicular sperm. Improved understanding of the genetic pathogenesis of SCO is essential for the development of prognostically relevant diagnostic tests in male infertility, and may ultimately enable development of novel therapeutic strategies. Additional diagnostic tests are also needed to inform prospective couples of the genetic implications for ART-conceived offspring.

ETS (E twenty-six) variant gene 5 (*ETV5*; alias ETS-related molecule) belongs to a family of transcription factors that share an evolutionarily conserved DNA-binding domain known as the ETS domain (15). This domain recognizes and binds target DNA in a sequence-specific manner at a purine-rich 5′-GGAA/T-3′ core sequence. ETS proteins regulate promoter activities of genes involved in many cellular functions, including cell growth, differentiation, migration, proliferation, and apoptosis. Global deletion of the *Etv5* gene in the mouse results in male infertility as a consequence of progressive lost of SSCs after the first wave of spermatogenesis (16–18). The loss of SSCs results in an SCO phenotype and azoospermia in adulthood (16, 17), thus defining the critical role for ETV5 in SSC self-renewal and sustaining spermatogenesis.

Within the mouse testis, ETV5 is localized to fetal Sertoli cells and to both Sertoli cells and germ cells in the neonatal testis (18). Studies using reciprocal transplantation of neonatal germ cells from wild-type and $Etv5^{-/-}$ testes into W/W^{v} recipient testes showed that ETV5 expression in neonatal germ cells is required for the initial establishment of the SSC pool and for homeostasis of SSC self-renewal and differentiation (19). ETV5 expression in Sertoli cells is critical for maintaining normal spermatogenesis (19).

Further investigations have suggested that ETV5 deficiency results in decreased RET expression, which impairs the GDNF-RET-GFRA1 signaling pathway (18). Studies using short interfering RNA knockdown in mouse SSC cultures revealed that ETV5 regulates several genes essential for SSC self-renewal, including Brachyury (*T*) and CXC chemokine receptor type 4 (*Cxcr4*) (20). Microarray analysis of *Etv5*-deficient Sertoli cells revealed a decrease in several chemokine-encoding genes, including C-c-motif ligand 9 *(Ccl9), Ccl7*, *Cxcl5*, and *Cxcl12*, suggesting that Etv5 regulates the production of these chemokines. Loss of Etv5 was shown to impair chemoattraction of SSCs toward Sertoli cells, thus reducing SSC migration to the stem cell niche (21).

The role of ETV5 in male fertility has been well illustrated in the mouse; however, its role in human male fertility has not been defined. In the present study, we localized ETV5 protein within the adult human testis and assessed the association between *ETV5* genetic variants and human male infertility. This study is the first to reveal the levels of *ETV5* genetic alterations in fertile and infertile men and its association with human SCO and NOA infertility.

MATERIALS AND METHODS

Defining ETV5 Localization in the Human Testis

Human testis biopsy from a vasectomized man who had undergone vasectomy reversal was obtained with consent and fixed as previously described (22). A rabbit polyclonal antibody raised against amino acids 121–220 of human ETV5 was used for the detection of the ETV5 protein (sc-22807; Santa Cruz). Immunofluorescence was performed as previously described (22) with the use of 4 μg/mL ETV5 antibody and 4 μg/mL donkey anti–rabbit Alexa 555 secondary antibody (Life Technologies). Testicular cell subtypes were identified based on nuclear profiles and relative position within the seminiferous epithelium.

SCO, NOA, and Fertile Men

Genomic DNA samples of infertile men were obtained from the Monash Male Infertility Repository (23, 24) and the Weill Cornell Male Infertility DNA Repository. Patients with chromosomal abnormalities (Y chromosome deletion (including gr/gr), Klinefelter syndrome, and other karyotypic abnormalities), past cryptorchidism, congenital absence of the vas deferens, and ejaculatory duct abnormalities were excluded. In addition, patients with conditions that