Published in final edited form as:

Fertil Steril. 2015 January ; 103(1): 44–52.e1. doi:10.1016/j.fertnstert.2014.09.021.

Genomic and Genetic Variation in *E2F1* in Men with Non-Obstructive Azoospermia

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Abstract

Objective—To identify gene dosage changes associated with non-obstructive azoospermia (NOA) using array comparative genomic hybridization (aCGH).

Design—Prospective study.

Patients—110 men with NOA and 78 fertile controls.

Settings—Medical School

Interventions—None

Main Outcome Measure—The study has four distinct analytic components: aCGH, a molecular karyotype that detects copy-number-variations (CNV), Taqman-CNV assays to validate CNVs, mutation identification by Sanger sequencing and histologic analyses of testicular tissues.

Results—A microduplication at 20q11.22 encompassing *E2F1* was identified in one of eight men with NOA analyzed using aCGH. CNVs were confirmed and an additional 102 men with NOA screened using Taqman CNV assays for a total of 110 NOA men analyzed for CNVs in *E2F1*. Eight of 110 (7.3%) NOA men had microduplications or microdeletions of *E2F1* that were absent in fertile controls.

Conclusions—*E2F1* microduplications or microdeletions are present in NOA men (7.3%). Duplications or deletions of *E2F1* occur very rarely in the general population (0.011%), but *E2F1*

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gene dosage changes, previously reported only in cancers, are present in a subset of NOA men. These results recapitulate the infertility phenotype seen in mice lacking or over-expressing *E2f1*.

Keywords

Azoospermia; *E2F1*; infertility; NOA; cryptorchidism; Array comparative genomic hybridization (aCGH); Copy number variations (CNV)

Introduction

Infertility affects approximately 15% of couples (1). Recognized genetic causes of severe spermatogenic failure include karyotype abnormalities, Y-chromosome microdeletions, systemic syndromes, and gene mutations. However, despite advances in molecular diagnostics, the etiology of most male infertility remains unknown or undiagnosed (2, 3). Elucidating the role of gene sequence variants, including single nucleotide polymorphisms (SNPs) and copy number variations (CNVs) defined by array comparative genomic hybridization (aCGH), provides a novel approach for identifying candidate fertility genes. Mouse models of these genes that recapitulate the phenotype allow investigators to demonstrate the causality of human gene defects affecting fertility.

This study focuses on E2F transcription factor-1 (*E2F1*), a gene encompassed by a CNV identified in an infertile cryptorchid man by aCGH. E2F1, encoded by a gene located on human chromosome 20, exhibits diverse and sometimes opposing functions (4). E2F1 both activates and represses the expression of genes involved in DNA damage and DNA repair, cellular differentiation, and autophagy. E2F1 may activate the cell cycle by regulating DNA synthesis and cell proliferation, and conversely, may induce apoptosis in both p53-dependent and p53-independent manners. Furthermore, depending upon the cellular context, E2F1 exhibits oncogenic and tumor suppressive activities. However, the final direction in which *E2F1* will lead the cell depends upon its genetic status or molecular background (5, 6). In mice, targeted deletion or overexpression of *E2f1* by transgenesis results in male infertility. Mice lacking *E2f1* exhibit increased tumor incidence and testicular atrophy with aging (>36 weeks old) (7, 8) as a result of spermatogonial cell loss, whereas Sertoli cells appear unaffected (9). Conversely, mice overexpressing *E2f1* are also infertile and exhibit testicular atrophy resulting from massive p53-independent apoptosis that results in germ cell, but not Sertoli cell, depletion (10, 11). Prolonged activation of *E2f1* mimics human carcinoma *in situ* (12). Given E2F1's role in the cell cycle, apoptosis, and infertility in mice, we sought to define the role of genomic and genetic changes in *E2F1* in human male infertility.

Materials and Methods

Selection of Study Population and Study Phases

This study was approved by the Institutional Review Board for the Protection of Human Subjects at Baylor College of Medicine. Supplementary Table 1 provides a summary of clinical data on both men with nonobstructive azoospermia (NOA) and fertile control patients. Infertile men with NOA were selected on the basis of a comprehensive infertility

evaluation including examination of medical history, physical examination, semen analysis, hormone analysis, karyotyping, and Y-chromosome microdeletion screening. Semen analysis (sperm count, motility, and morphology) was performed according to the World Health Organization (WHO) edition IV guidelines (1999) (13). The diagnosis of NOA was defined as the absence of sperm in two consecutive ejaculates and no evidence of genital tract obstruction. Of the infertile men evaluated, 14% had cryptozoospermia (rare sperm observed in the pellet following centrifugation of the semen) in at least one ejaculate; sperm were absent in their remaining samples. Eight men with NOA (7.2%) had a history of cryptorchidism corrected by orchidopexy. Fertile controls fathered one or more healthy children following less than twelve months of unprotected intercourse. No additional clinical data are available for the majority of this population; however, semen parameters were available for 5 fertile controls and were normal (63.5 ± 49.5 million/ml with a motility of $52.0 \pm 17.9\%$). Patients were evaluated according to the AUA Practice guidelines (53), and those with known causes of infertility were excluded from analysis, as were those with an abnormal karyotype or Y-chromosome microdeletions. Genomic DNA was isolated from 5ml of whole peripheral blood using the Qiagen Puregene DNA extraction kit (Valencia, CA) according to the manufacturer's protocol. A case-control study was designed that featured four distinct analysis components.

Phase One – array Comparative Genomic Hybridization (aCGH)

Commercially available genome-wide aCGHs ($3 \times 720K$ from NimbleGen, WI) were used to detect CNVs present in 8 infertile patients. Study and reference samples were labeled with Cy3 and Cy5, respectively, and were processed at the NimbleGen Service Lab (Iceland). Data were analyzed using SignalMap (Roche) and Nexus Copy Number (BioDiscovery) software. Clinical oligonucleotide-based aCGH testing was performed at SG using custom-designed arrays (SignatureChipOS versions 1 & 2, manufactured by Agilent, CA, and Roche NimbleGen, respectively) according to previously described methods (14, 15).

Phase Two - qPCR Validation

To validate aCGH findings, a TaqMan CNV assay for *E2F1* (Hs00576444_cn, Applied Biosystems (ABI), CA) was used. TaqMan CNV reactions were performed in triplicate using the FAM-dye-labeled assay for *E2F1* and VIC-dye-labeled RNaseP assay as a reference gene as previously described (16). Relative quantitative analysis to estimate copy number was performed using CopyCaller Software V1.0 (ABI, CA). Samples were run at least twice in independent assays to confirm results.

Phase Three – Single Nucleotide Polymorphism (SNP) Detection

Sanger sequencing was used to determine the presence of single nucleotide polymorphisms (SNPs) within the *E2F1* genes of the participants. For PCR, 50ng of gDNA was amplified using Phusion High-Fidelity PCR Master Mix with GC Buffer (New England Biolabs, MA). PCR products were purified using the ExoSAP-It kit (USB Scientific, OH) and sequenced on ABI 3730xlDNA analyzers with capillary electrophoresis and fluorescent dye terminator detection (Genewiz Inc., NJ). Data were analyzed using Mutation Surveyor software (Softgenetics Inc., PA).

Phase Four - E2F1 Detection in Testicular Samples

We evaluated the presence and localization of E2F1 protein in testicular tissues of men with NOA. Immunohistochemistry on testicular samples was performed on 5µm thick paraffin-embedded sections. The avidin-biotin-immunoperoxidase complex technique that uses diaminobenzidine (DAB) as a substrate was employed to generate a brown-colored polymeric oxidation product (Vector Labs, CA). Primary antibody for E2F1 (H-137, Santa Cruz Biotechnology, CA) was used at a concentration of 2µg/mL. Non-immune rabbit serum provided the negative control, and hematoxylin was the counterstain. For image preprocessing, segmentation, and measurement the Beer-Lambert law was applied to bright-field images of a control and patient (Table 1, #9) to roughly approximate the absorbance and, in turn, the amount of staining in each pixel of the image. Non-negative matrix factorization (17) was then used to differentiate the hematoxylin and DAB stains (18). With the use of the hematoxylin channel, 16 tissue tubules from each sample were segmented using a global threshold to identify non-tissue regions, and then these segmented regions were grown with a seeded watershed routine until they filled the entire image space. This approach produced over-segmentation in which single tubules were associated with multiple regions. To address over-segmentation, contiguous regions were manually merged into a single tubule, and tubules at the periphery of the image were discarded. Next, nuclei in the tubules were detected in the hematoxylin channel using a local threshold, and misshapen nuclei were discarded. Nuclear regions were dilated by a few pixels to roughly approximate the cell area. Finally, total intensity was measured in the DAB channel under these cell masks. With a manually set threshold based on the sum DAB intensity, the numbers of DAB expressing and non-expressing cells in the tubules were counted.

Statistical Analysis

Statistical analyses were performed using SPSS version 19 (IBM Corporation, Somers, NY) software with $p < 0.05$ considered significant. Fisher's exact test was used to determine the significance of *E2F1* CNV frequency as well as for comparisons between demographic categorical variables. A two-sided Kolmogorov-Smirnov test was run on the images of the control and patient samples to determine whether they were drawn from similar distributions. Student's t-test was used for continuous variable comparisons for patient demographic data. All confidence intervals (CIs) presented are at a 95% confidence level.

Results

Phase One – aCGH Reveals an E2F1 Microduplication in a Male with NOA

The eight men with non-obstructive-azoospermia (NOA) analyzed by aCGH displayed an average of 47.88 (95%CI, 36.3–58.4) CNVs per genome. After exclusion of regions lacking genes and areas of known genomic variation (benign CNVs), the average number of CNVs was 3.2 per patient (95%CI, 1.81–4.94). One NOA patient with a history of cryptorchidism corrected by orchiopexy displayed a discrete 138kb microduplication at chr20: 31.627.047–31.765.316 (UCSC-hg18) spanning 35 probes not found in any of the other 8 NOA men or 78 controls consisting of men with proven fertility or an unrelated birth defect analyzed by aCGH in our laboratory. This CNV encompassed five genes, *CBFA2T2*, *NECAB3*, *ACTL10*, *E2F1*, and *PXMP4*, and one uncharacterized open reading frame (*C20orf144*) (Fig. 1A). Of

these, only *E2F1* is associated with fertility in male mice in which both deletion and overexpression result in testicular atrophy (7–11). *CBFA2T2* is involved in acute myeloid leukemia (19), *NECAB3* is a Golgi protein possibly involved in regulating amyloid precursor protein metabolism and beta-amyloid generation (20), and *PXMP4* is a peroxisome protein with an anti-tumor action on prostate cancer (21). *ACTL10* has no known function. *E2F1* was selected as a candidate gene for further study of its role in human male infertility because of its known role in murine spermatogenesis (7–11).

Phase Two - qPCR Indicates that *E2F1* CNVs are Associated with NOA

To confirm the gain detected by aCGH and to define the incidence of this CNV in NOA men, an *E2F1* Taqman CNV assay was used. We evaluated 110 men with NOA with a mean age of 33.2 ± 8.0 years, including the 8 NOA men initially analyzed by aCGH, and compared these to 78 healthy fertile male controls with a mean age of 43.9 ± 11.2 years. Of the 110 NOA patients tested, 8 exhibited CNVs in *E2F1* (7.3%) (Fig. 1B). Four men with NOA had an *E2F1* microduplication (3 copies) and four had a microdeletion (1 copy) (Table 1). The calculated copy number for patients with a microdeletion was 0.98 ± 0.07 and for patients with microduplications was 2.64 ± 0.07 , both significantly different from patients with a normal copy number (1.94 ± 0.19 [$p=0.007$]). None of the 78 fertile controls tested (data not shown) had CNVs in *E2F1* with a calculated copy number of 1.97 ± 0.19 . In addition, three subjects with CNV regions smaller than 30 Mb (1.8–9.5 Mb) and encompassing *E2F1* were identified among 28,423 (0.011%) probands referred for clinical testing and tested by aCGH at Signature Genomic Laboratories (SG). One of these individuals had cryptorchidism. A query of the DECIPHER database (<https://decipher.sanger.ac.uk/>), showed that 9 of 39,620 patients in DECIPHER database had CNVs encompassing *E2F1* (22), with two of these men cryptorchid (23).

Phase Three – *E2F1* Genetic Variants (SNPs) are Detected in Men with NOA

Altered *E2F1* function can occur through gene mutation. Therefore, exons 2–7 of *E2F1* were sequenced. Variants were identified in exons 2 and 7 (Table 1). Two men with NOA and one fertile control with benign prostate hyperplasia had a nonsynonymous variant p.Gly393Ser (rs3213176) in the *E2F1* transactivation domain, representing a pRB binding domain that negatively regulates *E2F1* transcription (6). This somatic mutation, p.Gly393Ser, was described in a patient with non-small cell lung carcinoma of 79 studied, but did not correlate with tumorigenesis (24). This SNP is present with an allele frequency of 0.0193% in individuals tested by the 1000 Genomes project (<http://www.ncbi.nlm.nih.gov/variation/tools/1000genomes>). Another patient had a synonymous variant p.Leu415Leu (rs147013405). Bioinformatics assessment using PolyPhen-2 (25), SIFT (26), and Mutation Taster (27) predicted these variants to be non-pathogenic, and NetPhos-2 did not predict these variants to represent new phosphorylation sites (28). In contrast, one patient had a nonsynonymous variant in exon 2 (CycA/cdk2-binding domain), p.Ala102Thr (rs145741678), not present in any control. This sequence variant is predicted to cause disease by Mutation Taster and is a very rare variant present only in one individual of more than 2000 tested by the 1000 Genomes project, with an allele frequency of 0.005%. The large size of the 1000 Genomes database allowed us to obtain allele frequencies in the control population and circumvented the need to genotype a control population ourselves;

however, one limitation of this approach is that men in this control population could be infertile and skew the frequency in the control population. Parental DNA was not available for any of these patients; therefore, inheritance could not be determined.

Phase Four - Altered E2F1 Expression is Associated with Testicular Pathology

In fertile participants, E2F1 protein was consistently detected as strong brown DAB staining found in the nucleus of spermatogonia, early stage spermatocytes around the periphery of the tubule, and occasionally in Leydig cells, but not in spermatids or Sertoli cells (Fig 2A). The majority of testicular biopsies from patients with NOA displayed a pattern of E2F1 staining different from that displayed in biopsies of participants with normal spermatogenesis, in part because of the decreased number or absence of germ cells in which E2F1 was expressed (Fig 2C–F). Panels 2C–D represent the testicular biopsy of two patients with E2F1 microdeletion that showed decreased E2F1 staining. Panel 2C is patient #1 with Sertoli cell only exhibiting no staining, which is expected due to the absence of germ cells. Panel 2D is patient #4 with maturation arrest and hypospermatogenesis exhibiting a few E2F1 positive cells, perhaps because of their E2f1 microdeletion. Thus, gene dosage loss shows clear evidence of reduced protein expression in testis biopsies of NOA men. Panels 2E–F represent the testicular biopsies of two patients with E2F1 microduplications that show decreased E2F1 staining. Panel 2E is patient #6 and panel 2F is patient #8, both with hypospermatogenesis. Even though these patients had microduplications, an increase in the levels of E2F1 staining was not detected.

Surprisingly, patient #9, who has two copies of *E2F1* but a SNP pAla102Thr in the E2F1-CycA/cdk2 binding domain, appeared to have an increase in the amount of E2F1-positive cells (Fig 2B). To determine if pAla102Thr causes an increase in the number of cells that express E2F1, we compared the number of DAB (E2F1) positive cells (red circles, Fig. 3B,D) to unstained cells (blue circles, Fig. 3B,D) in 16 tubules. Computerized image analysis demonstrated that a control participant had significantly fewer ($p=1.5e-5$) DAB positive cells, with an average of 58.2% DAB positive cells (Fig. 3B shows 101/176 [57.4%]). Patient #9 had an average of 71.1% DAB positive cells (Fig. 3D shows 199/220 [79.6%]). SNP pAla102Thr, a possibly damaging variant, may be important in male infertility since it alters the interaction with the CycA/cdk2-binding domain required for cell cycle progression and leads to defective spermatogonial proliferation.

Discussion

Clinically relevant CNVs adversely affect neurologic, immunologic, and reproductive development, as well as cause other complex diseases. These adverse events result from changes in gene dosage, gene or protein function, or other molecular mechanisms (16, 29, 30). The presence of gene mutations underlying male infertility is widely discussed (31). However, excluding Y-chromosome microdeletions, CNVs resulting in gene dosage changes affecting male fertility have not been widely studied (16, 32–35). aCGH allows the identifications of gene dosage defects associated with male infertility. Using mouse models with similar defects, we recapitulated the infertility phenotype to prove causation beyond association. Our analysis using a high-resolution aCGH with 720,000 probes identified a

duplicated region in 20q11.22 in a man with NOA. This duplicated region encompassed one gene (*E2F1*) with a known function and one known to cause male infertility in mouse male models. Three additional genes with functions that are not fully defined (*CBFA2T2*, *NECAB3* and *PXMP4*) were duplicated, as well.

The gene identified in our study, *E2F1*, plays a crucial role in the control of the cell cycle (5, 6). In mouse models, *E2f1* deficiency or excess results in testicular atrophy caused by spermatogenic failure (7–11), making *E2F1* an appealing candidate gene for the study of male infertility. These existing mouse models demonstrated the importance of proper *E2F1* dosage in fertility. Our studies of infertile men with gene dosage changes in *E2F1* correlated with the studies using mouse models. Within our study cohort, an equal number of microdeletions and microduplications in *E2F1* were identified in men with NOA. The majority of patients with a microduplication of *E2F1* (3/4) displayed hypospermatogenesis (one was cryptozoospermic), suggesting that a microduplication may correlate with a less severe spermatogenic phenotype. Conversely, three of the four patients with *E2F1* microdeletions have a Sertoli cell only phenotype, the most severe spermatogenic defect. Our results suggest that *E2F1* is a dosage-sensitive gene critical for normal spermatogenesis.

The presence of microduplications or microdeletions in *E2F1* occurred in all ethnicities with the majority (50%) identified in Hispanic patients. However, the study groups are too small to allow stratification of the data by ethnicity thereby making it difficult to draw any conclusions. In addition, ethnicity was self-defined in our population and may not be completely accurate, particularly since Hispanics represent a mixture of European, African, and Native American heritages (36). Nevertheless, it is unlikely that CNVs in *E2F1* are specific to an ethnic or racial group or occur in a normal population.

A limitation of our study is the inability to demonstrate if the identified CNVs are *de novo* or inherited. Our IRB approved protocol specifies that the patients must be de-identified, thereby preventing us from contacting the patients to request further material or information from them or their family members. Many patients refuse to participate in fertility genetics studies unless we specify that they and their family will not be contacted after their sample donation. This reflects the patients' desire for their diagnoses to remain private. While it may be preferable to know whether a CNV is *de novo* or inherited because of the presumed dichotomy of phenotypic severity, *de novo* CNVs may not necessarily result in more severe phenotypes, and inherited CNVs exhibit substantial phenotypic variability. The 16p11.2 CNV is an excellent and commonly occurring example of the range of phenotypic variation possible in individuals harboring identical, inherited CNVs (37). In addition, biologic modifiers can impact phenotypic variation (38). Therefore, the status of a given CNV as either inherited or *de novo* does not necessarily inform disease severity. Additionally, since RNA was not available from these patients, we cannot determine whether epigenetic factors regulate E2F1.

None of our fertile controls had CNVs in *E2F1*. Importantly, the men in the control group were able to procreate within twelve months of unprotected intercourse and therefore had no medical reason to undergo a semen analysis. Accordingly we compared fertility-proven control men with men who were infertile due specifically either to non-obstructive

azoospermia (no sperm in the ejaculate) or cryptozoospermia (very few sperm in the ejaculate). These inclusion criteria narrow potential patient heterogeneity. Data from a private clinical database from Signature Genomic Laboratories showed that of 28,423 individuals tested (14, 15) only 3 individuals (0.011%) referred for clinical testing due to developmental delay and dysmorphic features had CNVs encompassing *E2F1*. However, these CNVs were much larger than those in our patients, ranging from 1.8–9.5 Megabases (Mb). An unrelated public database of CNVs, DECIPHER (<https://decipher.sanger.ac.uk/>), indicates that of the 39,620 patients with CNVs present anywhere within the genome, only nine have large CNVs encompassing *E2F1* ranging from 2.2–9.8Mb (0.023%). The frequency of *E2F1* CNVs in the men tested with NOA (7.3%) is significantly higher ($p < 0.007$) than the frequency of *E2F1* CNVs observed in population databases not selected for infertile males (0.011% to 0.023%). In addition, when the incidence of Y-chromosome microdeletions (8%) or structural chromosomal aberrations (up to 13%) in the infertile male population is considered, the frequency of CNVs in *E2F1* is likely clinically significant. While we focused on *E2F1* in our study, we acknowledge the possibility that other genes in conjunction with *E2F1* may be involved in these patients' infertility. However, a CNV or mutation in *E2F1* may represent the minimal insult necessary for male infertility. In our index patient, this was the only region present in the database of normal genomic variants remaining after removal of all benign CNVs.

One caveat of our study is that the index patient has cryptorchidism, although additional infertile men with CNVs in *E2F1* with normal testicular descent were identified. Cryptorchidism is a cause of azoospermia in adults with an incidence of 13% in unilateral cryptorchidism and 89% in untreated bilateral cryptorchidism (39). Orchidopexy performed before the age of 2 years minimizes germ cell loss, and when paternity is used as an index of fertility there is no significant difference in the ability or the time required to father a child between men with unilateral cryptorchidism (89.7%) and controls (93.7%) (40). However, there was a significant decrease in fertility in men with bilateral cryptorchidism (65.3%) (41). It is difficult to know if the spermatogenic failure in the index patient resulted only from his history of cryptorchidism or if it specifically resulted from *E2F1* microduplication. Importantly, of the eight men in the infertile cohort with a history of cryptorchidism, five did not have *E2F1* CNVs. Three cryptorchid males with *E2F1* microduplication have been described in the literature (23, 42), suggesting that *E2F1* may also play a role in testicular descent in men.

Since *E2F1* is required for male germ cell development, its translation is tightly regulated. Recent studies suggest these modulators include miRNAs (43–45). In normal human testis, *E2F1* mRNA is expressed in most germ cell types. However, only a subpopulation of spermatogonia and early primary spermatocytes near the edge of the tubule express *E2F1* protein, probably due to translational repression by the miRNA-17–92 cluster (44). In addition, high levels of miRNA-449 and miRNA-34b/c in the early meiotic phase were proposed to suppress *E2F* activity during the meiotic phase of spermatogenesis (43). The absence of *E2F1* in haploid germ cells suggests critical roles in mitosis. *E2F1* plays a pivotal role in the S-phase checkpoint by interacting with CycA/cdk2 binding-domains (functional *E2F1* domains encoded in exons-1–2, aa 67–108). This interaction causes dimerization,

protein phosphorylation and down-regulation of E2F1 activity during the S-phase (6). Cells with mutant E2F1 lacking the complete CycA/cdk2-binding-domain arrest in S-phase of the cell cycle and then undergo apoptosis (46). In contrast, an E2F1 mutant containing a truncated CycA/cdk2 binding-domain has a reduced binding that enables the cells to pass through the S-phase checkpoint at a reduced rate without driving the cells into apoptosis (47). We identified the mutation p.Ala102Thr in a Sertoli cell only and early meiotic arrest patient who exhibited significantly increased expression of E2F1 that could correlate with a down-regulation of entry into S-phase. Accordingly, this mutation could have a limited but detectable effect on CycA/cdk2. We cannot exclude the possibility that the increased number of E2F1 positive cells could reflect the relative depletion of unstained cells (spermatids) in the biopsy sample shown in Fig. 3, rather than by an actual increase in the number of cells stained with E2F1. Unfortunately, the majority of the biopsy samples are used for assisted reproductive technologies. Given the small amount of tissue available for research, no additional protein localization studies using immunohistochemistry could be performed. However, aberrant *E2F1* may cause testicular atrophy as a result of defective spermatogonial proliferation.

Microduplication of 20q11 leading to increased *E2F1* expression has been reported in colon (48), esophagus (49), melanoma (50), and prostate tumors (51). To our knowledge this is the first reported association of a microduplication or microdeletion of *E2F1* in non-cancer patients, emphasizing the range of actions of *E2F1* in complex diseases, including infertility. That gene dosage changes in *E2F1* (both microdeletions and microduplications) cause infertility in men is not surprising as there are many examples reported in the literature in which microdeletions and microduplications cause similar or identical phenotypes (52). Both events produce DNA rearrangements. An excellent example of this principle is demonstrated by the observation that both microdeletions and microduplications in the 2q23.1 region, encompassing the gene *MBD5*, are associated with autism (53, 54). Similarly to the current studies of E2F1 in spermatogenic failure, microduplication of 2q23.1 results in a slightly less severe phenotype than the reciprocal deletion (53). *De novo* CNVs are more likely to be causative of an abnormality (29), but inheritance could not be defined in these subjects. Regardless, the subjects' testicular phenotypes paralleled the histopathology observed in the mouse models (7–11) suggesting that genomic and genetic dosage changes in *E2F1* cause spermatogenic failure in a subset of infertile men.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Judy Choi, M.D. and John W. Weedon, M.D. (Baylor College of Medicine, Scott Department of Urology) for their help with sample collection. They did not receive any compensation for their role in this study.

Funding/Support: This study was supported in part by NIH grants from the National Institute of Kidney and Digestive Diseases 1R01DK078121 and K12 DK0083014 (Multidisciplinary K12 Urology Research Career Development Program-KURE) and from the Eunice Kennedy Shriver National Institute of Child Health and Human Development P01HD36289 to DJL. CJJ is a K12-KURE scholar. AWP is the recipient of the AUA Foundation Russell Scott Research Award grant. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. This study makes use of data generated by the DECIPHER

Consortium. A full list of centers who contributed to the generation of the data is available from <http://decipher.sanger.ac.uk> and via email from decipher@sanger.ac.uk and funding for the project was provided by the Wellcome Trust.

Role of the Sponsors: NIDDK, NICHD, Urology Care Foundation, DECIPHER, or PerkinElmer, Inc had no role in the design or conduct of the study; the collection, management, analysis, or interpretation of the data; or the preparation, review, or approval of the manuscript.

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Fig. 1. Infertile men with NOA display microdeletions, microduplications in *E2F1*

A) aCGH revealed a microduplication of 138kb at 20q11.22 (indicated by blue area) that encompasses the *E2F1* gene (highlighted red region) in a SCO-MA infertile patient (blue dots indicate probe position). B) Taqman-CNV analysis of NOA patients identified four patients with three copies of *E2F1* (green bars) indicating a microduplication and four NOA men with one copy of *E2F1* (red bars) indicating a microdeletion; the remaining patients had two copies of *E2F1* (normal, blue bar and data not shown).

Fig. 2. Immunohistochemical localization of E2F1 in human testicular tissue

Immunohistochemistry of testicular samples was performed to determine localization and expression of E2F1 protein (H137 antibody) by using DAB as a substrate to generate a brown-colored polymeric oxidation product that indicated protein localization. Hematoxylin was used as a counterstain. Image panels are at 200× magnification. A) Fertile participant showing E2F1 protein presence within the nucleus of spermatogonia (Sg), spermatocytes (Sp), and Leydig cells (Lc). No staining was present in spermatids (St) or Sertoli cells (Sc). B) Patient #9 (MA and SCO) with a SNP p.Ala102Thr in the CycA/cdk2 binding domain

(BD) has an increase in the number of E2F1 positive cells. C) Patient #1 with Sertoli cell only and *E2F1* microdeletion with no E2F1 staining in the testis. D) Patient #4 with maturation arrest and hypospermatogenesis, and *E2F1* microdeletion with only few cells E2F1 positives. E) Patient #6 with hypospermatogenesis, and *E2F1* microduplication with only few cells E2F1 positive. F) Patient #8 with hypospermatogenesis, and *E2F1* microduplication with only few cells E2F1 positive.

Fig. 3. Quantification of E2F1 positive cells in human testicular tissue

For Image preprocessing, the number of DAB expressing and non-expressing cells in the tubules were counted. Image panels are at 200 $\times$ magnification. A scale bar denoting 20 μ m in the image is shown. A) Fertile participant showing E2F1 protein presence within the nucleus of spermatogonia (Sg), spermatocytes (Sp), and Leydig cells (Lc). No staining was present in spermatids (St) or Sertoli cells (Sc). B) Image analysis based cell counting of a seminiferous tubule cross-section (green circle) of fertile male with DAB (E2F1) positive cells (red circles) and unstained cells (blue circles). C) Patient #9 (MA and SCO) with a

SNP p.Ala102Thr in the CycA/cdk2 binding domain (BD) has an increase in the number of E2F1 positive cells. D) Image analysis based cell counting of seminiferous tubule cross-section (green circle) from patient #9 with DAB (E2F1) positive cells (red circles) and unstained cells (blue circles).

Table 1

Characteristics of Men with NOA displaying CNVs and SNPs in E2F1

ID	Ethnicity	Testis Vol (ml)	CNV	SNP	Biopsy	Sperm Count	Cryptorchidism
1	Hispanic	14	1X	None	SCO	0	No
2	Black	16	1X	None	SCO	0	No
3	Asian	10	1X	None	SCO, MA and Leydig cell hyperplasia	0	Yes
4	Caucasian	16	1X	None	MA and Hypospermatogenesis	0	No
5	Hispanic	14	3X	None	SCO and MA	0	No
6	Hispanic	10	3X	None	Hypospermatogenesis	7 in pellet	No
7	Hispanic	16	3X	None	Hypospermatogenesis and Leydig cell hyperplasia	0	Yes
8	Middle Eastern	20	3X	p.Leu415Leu (Exon 7)	Hypospermatogenesis	0	No
9	Caucasian	NA	2X	p.Ala102Thr (Exon 2)	SCO and MA	0	Yes
10	Caucasian	NA	2X	p.Gly393Ser (Exon 7)	SCO and MA	0	No
11	Caucasian	18	2X	p.Gly393Ser (Exon 7)	MA	14 in pellet	No

E2F1 copy number variations (CNV) are indicated: 1X indicates copy number loss (microdeletion), 3X indicates copy number gain (microduplication), 2X indicates normal copy number. SNPs present in exon 2 and 7 are indicated. One fertile participant (F45) has a SNP in exon 7. Some data were not available (NA).