Reduced meiotic recombination on the XY bivalent is correlated with an increased incidence of sex chromosome aneuploidy in men with non-obstructive azoospermia

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Both aberrant meiotic recombination and an increased frequency of sperm aneuploidy have been observed in infertile men. However, this association has not been demonstrated within individual men. The purpose of this study was to determine the association between the frequency of recombination observed in pachytenic spermatocytes and the frequency of aneuploidy in sperm from the same infertile men. Testicular tissue from seven men with non-obstructive azoospermia (NOA) and six men undergoing vasectomy reversal (controls) underwent meiotic analysis. Recombination sites were recorded for individual chromosomes. Testicular and ejaculated sperm from NOA patients and controls, respectively, were tested for aneuploidy frequencies for chromosomes 9, 21, X and Y. There was a significant increase in the frequency of pachytene cells with at least one achiasmate bivalent in infertile men (12.4%) compared with controls (4.2%). Infertile men also had a significantly higher frequency of sperm disomy than controls for chromosomes 21 (1.0% versus 0.24%, P = 0.001), XX (0.16% versus 0.03%, P = 0.004) and YY (0.12% versus 0.03%, P = 0.04). There was a significant correlation between meiotic cells with zero MLH1 foci in the sex body and total sex chromosome disomy (XX + YY + XY) in sperm from men with NOA (r = 0.79, P = 0.036).

Keywords: azoospermia; sperm aneuploidy; ICSI; meiotic recombination; synaptonemal complex

Introduction

Meiotic recombination binds homologous chromosomes together with crossovers, thereby assisting in the proper segregation of homologs at the first meiotic division in spermatogenesis (Coop and Przeworski, 2007). Using genetic linkage analysis, aberrations in meiotic recombination, such as diminished frequency (Hassold et al., 1991; Thomas et al., 2000; Shi et al., 2001; Reish et al., 2004) and suboptimal location (Hassold et al., 1995; Lamb et al., 1997), have been suggested to impart a risk for non-disjunction and aneuploid gametes in humans as well as model organisms. Several studies have reported that infertile men have an increased frequency of aneuploid sperm (Moosani et al., 1995; Aran et al., 1999; Pang et al., 1999; Calogero et al., 2003) and this is particularly marked for infertile men with non-obstructive azoospermia (NOA) (Bernardini et al., 2000; Palermo et al., 2002; Martin et al., 2003). Men with NOA are now able to father children with the advent of intracytoplasmic sperm injection (ICSI) techniques (Palermo et al., 1992) following testicular extraction of sperm. However, it is also recognized that ICSI carries a significantly increased risk of producing aneuploid offspring (Martin, 1996; Van Steirteghem et al., 2002). Although relatively little is known about the genetic basis of aneuploidy, meiotic studies suggest that errors in recombination are a cause of aneuploid gametes (Hassold et al., 1991, 1995; Lamb et al., 1997; Thomas et al., 2000; Shi et al., 2001; Reish et al., 2004). Immunofluorescence methods that directly visualize important meiotic proteins have made possible the close examination of recombination events during meiosis (Barlow and Hultén, 1998; Tease et al., 2002; Sun et al., 2004a,b). Antibodies against SCP1 and SCP3 [synaptonemal complex (SC) proteins] mark the transverse and lateral elements of the SC, respectively; CREST (Calcinosis, Raynaud’s phenomenon, Esophageal dysfunction, Sclerodactyly, Telangiectasia) marks the
centromere, and MLH1 (mut L homolog 1, a mismatch repair protein) marks the recombination foci, allowing the precise identification of recombination foci along SCs during meiotic prophase. This assay, combined with centromere-specific multicolor fluorescence in situ hybridization (cenM-FISH), allows analysis of recombination distributions of individual chromosomes in human germ cells in great detail (Nietzel et al., 2001; Oliver-Bonet et al., 2003). Indeed, with these techniques, the first recombination maps for individual human chromosomes have been reported (Sun et al., 2004a, 2006b).

Using immunofluorescence techniques to examine meiotic recombination directly, we have determined that men with NOA have a variety of meiotic defects and a dramatic decrease in the frequency of meiotic recombination (Gonsalves et al., 2004; Sun et al., 2004a, 2005). We and others have also determined that the chromosomes most commonly observed to be achiasmate (with no recombination directly, we have determined that men with NOA have a variety of meiotic defects and a dramatic decrease in the frequency of meiotic recombination (Nietzel et al., 2001; Oliver-Bonet et al., 2003)). Indeed, with these techniques, the first recombination maps for individual human chromosomes have been reported (Sun et al., 2004a, 2006b).

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compare clustered binomial proportions (Donner and Klar, 1994). The correlation between MLH1 focus frequency and sperm aneuploidy for individual chromosomes was tested using a Spearman correlation coefficient. Similarly, the correlation between bivalents with no recombination foci and sperm aneuploidy was tested with a Spearman correlation coefficient.

Results

An example of pachytene SCs, with identification of individual bivalents and cenM-FISH signals in the same cell, is shown in Fig. 1. For the seven NOA patients, a total of 688 pachytene stage cells were analyzed; the overall mean frequency of autosomal MLH1 foci per cell was 48.4, with a range of 12–67 foci per cell (Table I). In all, 600 pachytene-stage spermatocytes were analyzed in controls (100 cells/donor) to determine the mean MLH1 focus frequency per cell for autosomes, with an overall mean of 50.7 foci (range: 32–63; Table I). Unlike previous studies (Sun et al., 2004a, 2005, 2007), there was no significant overall difference in autosomal recombination frequencies between NOA patients and controls ($P = 0.25$). However, the proportion of cells in NOA patients containing one or more autosomal SCs without an MLH1 focus (12.4%) was significantly higher than that observed in controls (4.2%; $P = 0.024$, Table I). An example of a pachytene cell with achiasmate bivalents is shown in Fig. 2.

The frequencies of achiasmate (non-crossover) bivalents for the sex chromosomes are presented in Table I. The frequency of MLH1 foci per cell in SCs 9 and 21 and of achiasmate bivalents are presented in Table II. Individual SCs for chromosomes 9 and 21 were identified in only four NOA patients (because FISH analysis of spermatocytes failed in three men). Compared with controls, there was a significantly increased achiasmate frequency for sex bivalents ($P = 0.03$) (data not shown) and bivalent 9 ($P = 0.001$) in NOA patients.

Sperm aneuploidy frequencies for chromosomes 9, 21, X and Y were assessed by FISH analysis. More than 50,000 spermatozoa were scored for each group (Table III). Disomy frequencies were significantly elevated in NOA patients compared with controls for chromosome 21 ($P = 0.001$), XX ($P = 0.004$) and YY ($P = 0.04$).

There was a significant correlation between the frequency of pachytene cells with zero MLH1 foci in the sex body and the frequency of YY disomy ($r = 0.86$, $P = 0.014$) and the frequency of total sex chromosomal disomy (XX + YY + XY, $r = 0.79$, $P = 0.036$) in testicular sperm from NOA men.

Discussion

We and others have previously demonstrated that infertile patients with NOA have a significantly reduced frequency of recombination in pachytene spermatocytes compared with controls (Gonsalves et al., 2004; Sun et al., 2004a, 2007). However, in this study, we found no significant decrease in mean recombination frequency in NOA patients compared with controls, similar to two other recent studies (Ma et al., 2006a; Topping et al., 2006). This discrepancy is likely to be related to differences in subject populations. Indeed, NOA patients in this study were only a subset of our total NOA

| Controls Autosomal MLH1 foci per cell | % cells with an autosomal bivalent containing 0 MLH1 | % cells with 0 MLH1 foci in the sex body |
|--------------------------------------|-------------------------------------------------|----------------------------------------|
| Mean | Range | 5 | 10.0 |
| 1 | 53.2 | 37–62 | 5 |
| 2 | 49.2 | 32–60 | 4 |
| 3 | 49.9 | 33–61 | 7 |
| 4 | 49.9 | 40–60 | 6 |
| 5 | 50.7 | 38–59 | 2 |
| 6 | 51.5 | 37–63 | 1 |
| Mean | 50.7 | 32–63 | 4.2 |

| NOA Autosomal MLH1 foci per cell | % cells with an autosomal bivalent containing 0 MLH1 | % cells with 0 MLH1 foci in the sex body |
|---------------------------------|-------------------------------------------------|----------------------------------------|
| Mean | Range | 7 | 14.9 |
| 7 | 45.2 | 20–54 | 23 |
| 8 | 48.9 | 23–61 | 7 |
| 9 | 42.7 | 15–61 | 16 |
| 10 | 55.7 | 44–65 | 0 |
| 11 | 45.0 | 12–57 | 18 |
| 12 | 48.4 | 27–62 | 18 |
| 13 | 53.2 | 25–67 | 3 |
| Mean | 48.4 | 12–67 | 12.4* |

* $P = 0.024$ compared with controls.

Figure 1: (Upper) Human pachytene spermatocyte with SCs shown in red, centromeres in blue and MLH1 foci in yellow. (Lower) Subsequent cenM-FISH analysis permits identification of individual chromosomes so that recombination (MLH1) foci can be analyzed for each SC.
population and were selected for inclusion because they had sperm present in the testes to allow for comparison of meiotic recombination and sperm aneuploidy. Thus, these NOA patients likely had fewer meiotic errors than NOA patients without sperm, as they completed meiosis and developed sperm. Despite this, these NOA patients had a significantly increased frequency of pachytene cells with at least one bivalent with no recombination. These bivalents are at high risk of producing aneuploid gametes, because there is no crossover to ensure that homologous chromosomes remain tethered and correctly oriented on the metaphase plate for proper segregation.

Analysis of individual chromosomes demonstrated that NOA patients had a significant decrease in the frequency of recombination in the sex chromosomes, and an increase in the frequency of bivalents without a recombination focus for chromosome 9 and the sex chromosomes. Once again, this demonstrates the susceptibility of the sex chromosome pair to a lack of recombination.

The frequency of sperm disomy was significantly increased in NOA patients compared with controls for chromosomes 21, XX and YY. The frequency of XY disomy was 2-fold higher than in controls, but did not reach statistical significance. Other studies have also demonstrated that testicular sperm in NOA patients have a significantly increased frequency of disomy compared with controls, an effect most pronounced for the sex chromosomes (Levron et al., 2001; Burello et al., 2002; Martin et al., 2003). It is interesting that a significant increase in the frequency of XX and YY disomy was found, since these are derived from meiosis II errors, and involve the malsegregation of sister chromatids. Since recombination occurs at meiosis I, an obvious question arises: how can altered recombination be associated with meiosis II-derived disomies? One potential explanation is that aberrations in crossovers disrupt sister chromatid cohesion, which could lead to the premature separation of sister chromatids at meiosis I (Hassold and Hunt, 2001). Subsequently the two sister chromatids could travel to the same pole in anaphase, resulting in a disomic gamete (Hassold and Hunt, 2001; McDougall et al., 2005).

The most novel aspect of this study is that it is the first to demonstrate, in a population of infertile men, that a correlation exists between altered recombination and meiosis II-derived disomies. This highlights the importance of understanding the molecular mechanisms underlying meiotic recombination and chromosome segregation, as disruptions in these processes can have significant consequences for reproductive health.

### Table II. Analysis of MLH1 focus frequencies for chromosomes 9 and 21.

|        | SC9 | SC21 | SC9 | SC21 |
|--------|-----|------|-----|------|
| Controls | 2.44 (85) | 0.99 (85) | 0.0 | 4.7 |
| NOA*    | 2.25 (92) | 0.96 (92) | 0.0 | 5.4 |
|        | 2.35 (48) | 0.92 (48) | 0.0 | 8.3 |
|        | 2.24 (143) | 0.84 (143) | 0.0 | 16.8 |
| Mean   | 2.34 | 0.95 | 0.0 | 7.4 |

*Four NOA patients had information available for chromosomes 9 and 21.

|        | SC9 | SC21 | SC9 | SC21 |
|--------|-----|------|-----|------|
| SC9    | 0.97 (92) | 0.96 (156) | 3.1 | 9.0 |
| SC21   | 0.89 (129) | 0.97 (140) | 2.8 | 4.3 |
| Mean   | 0.94 | 0.94 | 2.9 | 7.3 |

### Table III. Aneuploidy frequency in spermatozoa for chromosomes 9, 21 and the sex body.

|        | XX | YY | XY | 9 | 21 |
|--------|----|----|----|---|----|
| Controls | 0.03 | 0.05 | 0.29 | 0.06 | 0.58 |
| NOA     | 0.04 | 0.01 | 0.15 | 0.16 | 0.18 |

*P = 0.04 compared with controls.

|        | XX | YY | XY | 9 | 21 |
|--------|----|----|----|---|----|
| SC9    | 0.21 | 0.06 | 0.13 | 0.21 | 0.56 |
| SC21   | 0.32 | 0.29 | 0.56 | 0.19 | 0.71 |
| Mean   | 0.32 | 0.29 | 0.56 | 0.19 | 0.71 |

|        | XX | YY | XY | 9 | 21 |
|--------|----|----|----|---|----|
| SC9    | 0.12 | 0.09 | 0.45 | 0.38 | 1.43 |
| SC21   | 0.29 | 0.41 | 1.40 | 1.40 | 1.97 |
| Mean   | 0.29 | 0.41 | 1.40 | 1.40 | 1.97 |

*P = 0.04 compared with controls.

*P = 0.004 compared with controls.

*P = 0.001 compared with controls.
between meiotic recombination and testicular sperm aneuploidy in the same individual. A low frequency of meiotic recombination in the sex chromosomes of NOA patients was significantly correlated with a high frequency of YY disomy and total sex chromosomal disomy in their sperm. A prior study of one individual (Ma et al., 2006b) found that an absence of recombination in the sex chromosomes was associated with an extremely high frequency of sex chromosomal aneuploidy in testicular sperm. In our prior study of fertile donors, we found no correlation between recombination and aneuploidy (Sun et al., 2008). These fertile men actually had a lower frequency of sperm aneuploidy than that observed in previous control populations. We hypothesized that these fertile men did not reach the threshold of abnormality to demonstrate a correlation. The current study demonstrates the correlation between a lack of meiotic recombination and sperm aneuploidy for sex chromosomes in infertile NOA patients. The sex chromosomes have consistently been shown to be the most susceptible to both recombination errors (Gonsalves et al., 2004; Codina-Pascual et al., 2006; Sun et al., 2006a) and sperm aneuploidy (Shi et al., 2001; Ma et al., 2006b), and we have observed this linkage within individuals for the first time.

A number of studies have shown that aberrant meiotic recombination in normal women is associated with the production of an aneuploid child (Hassold and Hunt, 2001; Thomas et al., 2001; Laurent et al., 2003). We were not able to directly demonstrate this association in normal men (Sun et al., 2008), perhaps because a stringent male pachytene checkpoint eliminates spermatocytes with meiotic abnormalities. Studies in a number of model organisms have demonstrated evidence for a pachytene checkpoint that responds to defective meiotic recombination and/or synopsis in spermatocytes in a p53-dependent or -independent manner (Odorioso et al., 1998; Cohen and Pollard, 2001; Hunt and Hassold, 2002). There is also evidence for a spindle assembly checkpoint which responds to chromosome kinetochores that fail to attach properly (Woods et al., 1999; Sluder and McCollum, 2000). Our study demonstrates that infertile men with dramatic meiotic abnormalities can succumb to the meiotic checkpoint (with loss of cells and consequent infertility), but may also escape the checkpoint, leading to an increased risk of aneuploidy.

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