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Microsatellite instability and defects in mismatch repair proteins: a new aetiology for Sertoli cell-only syndrome

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Microsatellite instability is characteristic of certain types of cancer, and is present in rodents lacking specific DNA mismatch repair proteins. These azoospermic mice exhibit spermatogenic defects similar to some human testicular failure patients. Therefore, we hypothesized that microsatellite instability due to deficiencies in mismatch repair genes might be an unrecognized aetiology of human testicular failure. Because these azoospermic patients are candidates for testicular sperm extraction and ICSI, transmission of mismatch repair defects to the offspring is possible. Seven microsatellite loci were analysed for instability in specimens from 41 testicular failure patients and 20 controls. Blood and testicular DNA were extracted from patient and control specimens, and amplified by PCR targeting seven microsatellite loci. DNA fragment length was analysed with an ABI Prism 310 Genotyping Machine and GeneScan software. Immunohistochemistry was performed on paraffinized testis biopsy sections and cultured testicular fibroblasts from each patient to determine if expression of the mismatch repair proteins hMSH2 and hMLH1 was normal in both somatic and germline cells. Results demonstrate that microsatellite instability and DNA mismatch repair protein defects are present in some azoospermic men, predominantly in Sertoli cell-only patients ($P < 0.01$ and $P < 0.05$ respectively). This provides evidence of a previously unrecognized aetiology of testicular failure that may be associated with cancer predisposition.

Key words: azoospermic/ICSI/microsatellite instability/mismatch repair proteins/Sertoli cell-only

Introduction

Male factor abnormalities account for 30–50% of all infertility cases (Lipshultz and Howards, 1997). Approximately 30–40% of men with abnormal sperm production do not have a detectable cause. It has been suggested that genetic alterations may be involved in a significant percentage of all severe cases (Rucker *et al*., 1998).

ICSI is a reproductive technology which, in conjunction with testicular sperm retrieval (TESE), is used to overcome severe male infertility to help couples achieve an otherwise impossible pregnancy (Palermo *et al*., 1992). This technique bypasses the natural barriers that would normally block fertilization by defective sperm. Thus, the widespread use of TESE–ICSI for the management of idiopathic male infertility has theoretical concerns. The long-term genetic consequences in the offspring conceived by these techniques are largely undefined and the transmission of undesirable genetic traits remains a possibility (Tripp *et al*., 1997; Matzuk and Lamb, 2002).

Organisms have evolved a number of mechanisms to minimize genetic defects by ensuring high-fidelity transmission of DNA from one generation to the next. DNA polymerase proofreading activity corrects errors arising during DNA replication. For the errors that are not corrected, the DNA mismatch system (MMR) is partially responsible for their repair (Loeb and Kunkel, 1982).

In humans, MMR is primarily mediated by the MutS yeast homologues (hMSH2, hMSH3 and hMSH6), which subsequently interact with the MutL homologues (hMLH1 and PMS2), ultimately recruiting endo- and exonucleases, as well as other proteins involved in DNA replication/repair that lead to the repair of the mismatched region. The hMSH2–hMSH6 heterodimers are mainly involved in mismatch recognition, whereas hMSH2–hMSH3 dimers can play a role in the repair of insertion–deletion loops. The somewhat redundant function of hMSH3 and hMSH6 highlights the importance of hMSH2 for the maintenance of genomic integrity. Similarly, hMLH1 is thought to have a unique role in MMR. hMLH1–PMS2, hMLH1–PMS1 and hMLH1–hMLH3 heterodimers occur, but only the former set of dimers (hMLH1 and PMS2) is proven to be involved in MMR (Jiricny and Nystrom-Lahti, 2000; Lipkin *et al*., 2000).

Studies in bacteria, yeast and rodents indicate that the genes encoding MMR enzymes are also required for normal meiosis (Bocker *et al*., 1999; Geeta Vani *et al*., 1999). In fact, mice with either targeted deletions or truncated mutations of MMR and MMR-associated proteins exhibit fertility disorders similar to those observed in humans. Conditions ranging from complete absence of spermatogenesis (*brac2* mutant mice) to morphologically abnormal sperm production (*pms2*-deficient mouse) or meiotic arrest (*mlh1*, *atm*, *mlh5* and *mlh3* null mice) occur (Baker *et al*., 1995; Barlow *et al*., 1996; Edelmann *et al*., 1996, 1999; Rotman and Shiloh, 1998; Lipkin *et al*., 2000). Likewise, the well-known human genetic disorder ataxia telangiectasia, which is caused by *atm* mutations, is also characterized by infertility (Barlow *et al*., 1996).

Table I. Microsatellite loci tested to determine genomic stability, their characteristics and the corresponding primers used for PCR amplification

Microsatellite	Type	Chromosome	Forward primer	Reverse primer	Associated gene
BAT-26	$(A)_n$	2	TGACTACTTTTGACTTCAGCC	CCCAATTTTTACAACTAACCAA	MSH2 gene
BAT-40	$(A)_n$	1	ATTAACTTCCTACACCACAAC	GTAGAGCAAGACCACCTTG	3β-HSD gene
D2S123	$(CA)_n$	2	ACATTGCTGGAAGTTCTGGC	ACCATAGGTTCAGTCTTTCC	–
D17S250	$(CA)_n$	17	GCTGGCCATATATATATTTAAACC	CCAAATTTATATATATACCGGTCG	–
D18S58	$(CA)_n$	18	GCTCCCGGCTGGTTTT	TTCAAGGACGCTAAAGGTCGG	–
D19S49	$(CA)_n$	19	GTGTTGTTGACCTATTGCAT	TACGTTATCCAGTTGTTGTG	–
AR, exon 1	$(CAG)_n$	X	AGGCACCCAGAGGCCGCGAGCGCAG	GAAGGTTGCTGTTCCTCATCCAGG	Androgen receptor gene

DNA repair defects such as these are a known cause of genomic instability (Jiricny and Nystrom-Lahti, 2000). DNA microsatellite analysis provides an important tool to assess genomic instability in malignancies and in normal tissues (Mao *et al*., 1994).

To date, human studies have not focused on the expression pattern of all MMR proteins during male germ cell differentiation; in rodents, hMSH2 and hMLH1 are expressed during spermatogenesis (Geeta Vani *et al*., 1999; Richardson *et al*., 2000). In mice, hMSH2 shows increased expression until the zygotene spermatocyte with reduced expression in the later stages of spermatocyte development. Trace levels of expression are present until the spermatid stage. hMLH1, on the other hand, shows a constitutive and regular pattern of expression during all stages of spermatogenesis until the spermatid stage. Both proteins exhibit nuclear localization in both rodents and humans, as determined by immunohistochemical analyses in other human tissues (not testis) (Simpkins *et al*., 1999).

Defects in some MMR proteins have systemic effects, such as enhanced cancer susceptibility in both null mice and humans (Liu *et al*., 1996; Dunlop *et al*., 1997; Prolla *et al*., 1998; Jiricny and Nystrom-Lahti, 2000). It is unknown whether patients with pathological testicular phenotypes similar to the ones observed in mice with defective DNA repair proteins exhibit microsatellite instability and are predisposed to develop tumours at an early age. Moreover, it is unknown whether the children conceived through TESE–ICSI are inheriting such a predisposition.

The current study tested the hypothesis that genomic instability is associated with spermatogenic failure in humans.

Materials and methods

Sample selection

Fifty azoospermic men undergoing testicular biopsy were recruited for this study with the approval and oversight of Baylor College of Medicine's Institutional Review Board for Human Subjects. Subjects gave informed consent. The mean age of all patients was 33 years. Of these patients, 30 underwent karyotype analysis for Y chromosome microdeletions. Two patients presented with karyotype abnormalities and two others with Y chromosome microdeletions.

Testicular specimens were collected and divided into three portions. One tissue fragment was fixed in Bouin's solution, paraffinized and stained for routine histological examination. Based on standard pathological qualitative guidelines, each biopsy was classified as Sertoli cell-only (SCO; 19 patients), maturation arrest (10 patients), hypospermatogenesis (12 patients) or normal, but obstructed, spermatogenesis (nine patients). A second portion of the sample (from the same biopsy side) was used for DNA extraction. The remaining portion was cultured *in vitro* to establish a primary testicular fibroblast culture.

Peripheral blood was collected from each patient and used for DNA purification.

Snap-frozen testicular and liver samples were obtained from 11 fertile deceased men immediately after death and stored at –70°C prior to DNA extraction to avoid tissue degradation and DNA fragmentation. As these men showed normal testis histology and no evidence of liver disease they were considered suitable controls. Additionally, fresh preputial tissue was collected from 10 healthy children undergoing circumcision, and cultured *in vitro* to obtain primary fibroblast cultures.

DNA extraction

Testicular and liver genomic DNA was isolated using the Wizard® Genomic DNA purification kit (Promega, Madison, WI, USA) following manufacturer protocol. Blood genomic DNA was prepared from peripheral blood leukocytes using Puregene™ DNA isolation kit (Gentra Systems, Inc., Minneapolis, MN, USA) as recommended. DNA concentrations were determined by standard spectroscopic measurements. Samples were stored at –20°C in Tris-EDTA buffer.

DNA amplification

Seven microsatellite loci located on different chromosomes were amplified from genomic DNA by PCR. According to the 'International Workshop on Microsatellite Instability and RER Phenotypes in Cancer Detection and Familial Predisposition' guidelines, analysis of a panel of at least five microsatellite loci is recommended to establish whether instability is present (Boland *et al*., 1998). Since microsatellite stability is believed to be maintained by several proteins, each specific for a certain type of error, analysis of different types of repeats known to be susceptible to instability should be performed. Thus, loci selected consisted of two mononucleotide tandem repeats (BAT-26 and BAT-40), four dinucleotide tandem repeats (D2S123, D17S250, D18S58, D19S49) and one trinucleotide repeat locus [within exon 1 of the androgen receptor (AR)]. Corresponding primer sequences used for PCR are described in Table I. Each of the forward primers synthesized by PE Biosystems (Foster City, CA, USA) was fluorescent-labelled with 6-FAM (D2S123, AR and D17S250), HEX (D18S58 and D19S49) or NED (BAT-26 and BAT-40). The reverse-oriented, non-labelled primers were supplied by Gibco (Life Technologies; Grand Island, NY, USA).

BAT-26/D2S123/D18S58 and BAT-40/D17S250/D19S49 loci were amplified by triplex PCR; AR exon 1 amplification was performed by single PCR. Triplex amplification of testicular, liver and blood DNA was carried out following PE Biosystems multiplex PCR recommended protocol. AR amplification was performed as previously described (Marcelli *et al*., 2000).

Fragment analysis and determination of microsatellite instability

PCR products were run on an ABI Prism 310 sequence analyser following the manufacturer's recommended protocol (Perkin Elmer, USA). Briefly, PCR products were separated by high-resolution fluorescent electrophoresis and the length of individual fragments determined with GeneScan software by comparison with the fluorescent-labelled internal size marker ROX-500 (PE Biosystems). Genomic DNA were assayed in two to four independent PCR amplifications and electrophoresis runs to verify reproducibility.

The elution pattern for paired blood and testis PCR fragments was compared and the length of the polymorphic repeats determined for all the loci. Normally, when genomic stability prevails, no difference is noted between the repeat lengths for the paired blood and testis samples, and these lengths are consistent throughout all tissues in the body. Thus, microsatellite instability is present when different sized alleles are noted in the same individual for blood and testicular DNA, for at least one of all the loci tested.

Primary testicular fibroblast culture

Fresh testicular and foreskin specimens were aseptically collected, plated and maintained in Dulbecco's modified Eagle medium, supplemented with 10%

fetal bovine serum and 2% penicillin–streptomycin (all provided by Gibco–Life Technologies; Grand Island, NY, USA), following standard protocols in order to set up fibroblastic cultures. Immunohistochemistry analysis was performed on the cultured fibroblasts at the second cell passage.

Immunohistochemistry analysis

Fibroblastic cultures in the exponential phase of growth were fixed on slide chambers with cold acetone for 10 min. Paraffin-embedded testicular 4 μm sections were assayed by immunohistochemistry using the Rabbit ABC Staining System kit following manufacturer's recommendations (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Affinity-purified rabbit polyclonal antibodies against hMSH2-N, hMLH1-N and hMLH1-C were used (Santa Cruz Biotechnology). The corresponding blocking peptides (Santa Cruz Biotechnology) were combined as recommended with each of the mentioned antibodies for neutralization and used as negative controls. As a positive control, an affinity-purified rabbit polyclonal antibody raised against c-FOS (Santa Cruz Biotechnology) was used. The concentration of primary antibodies was 5 μg/ml.

Following staining, slides were allowed to rest for 30 min at room temperature, and then microscopically examined at ×100, ×200 and ×400 magnification, to determine the pattern and localization of MMR enzyme expression. MMR expression was considered normal when the expression pattern (cell specificity and cellular localization) was nuclear and similar to that observed for the rodent testis (for details, see Introduction).

Statistical analysis

Histopathological states (maturation arrest, hypospermatogenesis, SCO and normal) were analysed with respect to those displaying and not displaying instability using a χ^2-test. Instability frequencies for the various histopathological states were similarly analysed. Fisher's one- and two-sided exact tests were used to define the significance of the mismatch repair defects of the individual pathologies. $P < 0.05$ was considered significant.

Results

To determine whether testicular failure patients present a higher incidence of genomic instability than men with normal testicular histology, seven microsatellite loci lengths were compared between paired blood and testis DNA samples from 41 azoospermic patients (presenting with SCO, maturation arrest or hypospermatogenesis) and 20 controls (11 deceased men and nine patients with obstructed azoospermia, all with normal testicular histology), as shown in Figure 1A and B. Although results were extremely consistent between different amplifications and fragment analyser runs (Figure 1A), of the 427 total loci tested for instability, successful amplification and fragment analysis could not be obtained for 8% of all loci, possibly due to DNA degradation. Therefore, the frequency of instability was also determined by a comparison of the number of loci affected to the total loci analysed for each of the histological categories.

The incidence of microsatellite instability was significantly related to testicular pathology ($P < 0.01$). The number of unstable DNA molecules present in each patient's sample was not assayed, as that type of quantitative analysis is not usually performed or required to determine the presence of microsatellite instability.

SCO patients exhibited a significant increase in the percentage of patients with microsatellite instability ($P = 0.007$) and the overall instability frequency ($P = 0.007$), as compared to all patients and controls with germ cells in the testis (Figure 1C). In addition, 10.5% of the SCO patients presented high microsatellite instability (H-MSI), with more than two of the loci analysed showing instability. Conversely, H-MSI was not observed in any controls or maturation arrest patients. It was present in 8.3% of the hypospermatogenesis patients (results not shown).

To define a potential cause for the observed instability, immunohistochemical analysis of hMSH2, hMLH1-N and hMLH1-C was used to characterize MMR protein expression in the testicular failure patients. These two proteins were chosen because of their essential role in MMR repair and meiosis, as well as for their known requirement for the maintenance of microsatellite stability in humans (Liu *et al*., 1996). Moreover, due to the known cell-specific pattern of expression in the rodent testis, these two proteins were expected to be present in the testicular sections of all the testicular failure conditions. To assess both the germinal and somatic cells, histological testicular sections and fresh, in-vitro cultured testicular fibroblasts from the same infertile patients were analysed respectively. Early cell senescence, perhaps due to cell culture shock or mitotic clock restrictions, prevented immunohistochemical analysis of several patients' fibroblasts. Additionally, testicular sections were not available from some of the testicular failure patients due to the small biopsy size. Thus, the pattern of expression of hMSH2 and hMLH1 in the germ cell lines of a few patients could not be assessed. A total of 25 patients was analysed for hMSH2 and hMLH1 somatic expression, of which 10 presented with SCO, five with maturation arrest, five with hypospermatogenesis and five with normal histology. Ten normal fibroblastic cultures (in-vitro cultured from foreskin samples of babies undergoing circumcision) provided additional normal controls. Nineteen patients were assessed for MMR protein expression in testis sections, of which eight presented SCO, six maturation arrest, one hypospermatogenesis and four normal histology.

The incidence of MMR protein expression abnormalities was significantly related to testicular pathology, being particularly increased in SCO patients ($P < 0.05$). The abnormal patterns observed in the male infertility patients consisted of a complete absence of expression of either hMSH2 and/or hMLH1, abnormal cellular localization of hMSH2 and/or hMLH1, and changes in immunoreactivity for hMLH1-N and hMLH1-C, suggesting truncation of the protein or other type of protein modification. Nevertheless, since immunohistochemistry is not a quantitative technique, this last observation must be confirmed by a different approach. No abnormal expression patterns were observed for any of the controls (results not shown). Results obtained (total and individual defects observed) are presented in Figure 2A and B. Figure 2B shows examples of the MMR expression defects found in azoospermic men assessed by immunohistochemical analysis.

Four of the azoospermic men included in this study presented a-priori structural chromosomal abnormalities not known to be related to either microsatellite instability or MMR abnormalities. These patients did not show any microsatellite instability between their blood and testis samples for all the loci examined and exhibited normal MMR protein expression patterns.

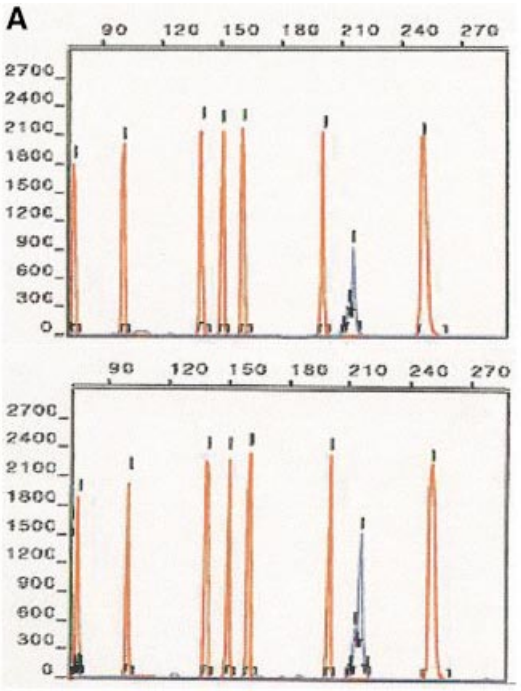
Discussion

ICSI has revolutionized the reproductive medicine field. Ten years ago, before the development of TESE–ICSI, most azoospermic men were simply unable to conceive a child. Currently, with the application of these techniques many men with the most severe forms of male infertility can experience fatherhood. These infertile men will now transmit their genes to their offspring through a conception only possible with ICSI. Genetic alterations have long been recognized as potential causes for some cases of human male infertility (Tripp *et al*., 1997; Matzuk and Lamb, 2002). Potentially, undesirable genetic phenotypes that are normally prevented by natural barriers to conception by defective sperm are now transmitted to the offspring of these azoospermic men.

With the advent of mouse embryonic stem cell gene targeting technology, we have witnessed an explosion in the identification of new genes affecting spermatogenesis. Whether these genetic defects

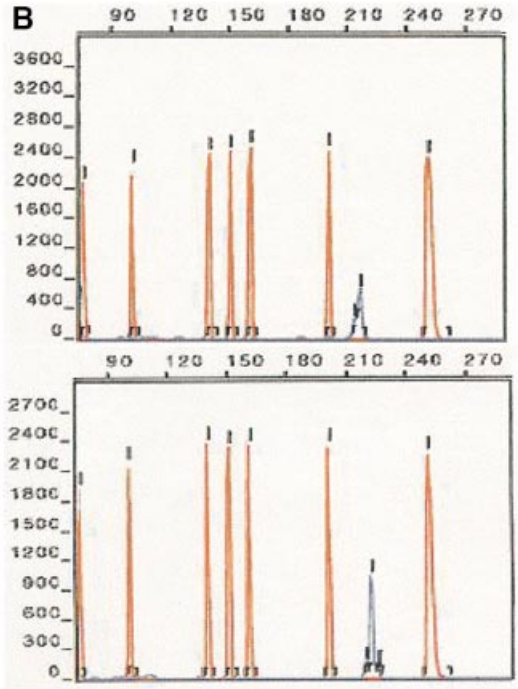
identified in mice are associated with human male infertility remains to be proven. One such group of genes required for male fertility encodes the proteins of the MMR system. Therefore, we hypothesized that defects in MMR might be the cause of some types of testicular failure. By analogy to some cancer patients and to mice models, transmission of these defective MMR genes to the offspring through TESE–ICSI may have profound systemic manifestations. Over 20 years ago, researchers suggested that mutations in genes required for DNA repair might result in the meiotic defects found in some infertile men (Chaganti and German, 1979). In fact, the human genetic disorder ataxia telangiectasia, caused by mutations in the DNA repair atm gene, is characterized by infertility (Barlow *et al*., 1996). Of note, the incidence of infertility in other DNA repair-deficient conditions, such as human non-polyposis colon cancer (HNPCC), is currently unknown. HNPCC is not usually diagnosed until the patients reach their early to mid-forties. Therefore, HNPCC patients with MMR germline mutations may experience a progressive decline in fertility with ageing prior to cancer onset. Thus, they may be able to conceive and pass on these gene defects before the physiological consequences of a defective MMR system are recognized (cancer).

Because there are no sperm in the ejaculate of azoospermic patients, testis biopsies provided the tissue to assess testicular DNA, including the germ cells. Since testicular genomic DNA is extracted from a



Minutes	Size	Peak Height
16.97	209.73	56
17.05	212.61	318
17.09	213.7	193
17.14	215.53	950

Minutes	Size	Peak Height
16.86	209.82	95
16.95	212.72	538
16.98	213.69	345
17.03	215.54	1522



Minutes	Size	Peak Height
16.95	212.72	273
17.04	215.55	658

Minutes	Size	Peak Height
17.03	218.2	168
17.12	221.25	1038
17.2	224.21	121

Figure 1A, B. Legend on facing page.

heterogeneous mixture of cell types with several embryonic derivatives (and the small biopsy fragments provided for basic research investigators are not sufficient for tissue dissection), it is important to ensure a representative sampling of all testicular cell populations present in the PCR-amplified DNA for analysis. Therefore, large pool PCR followed by fragment analysis was used. Microsatellite instability determination was performed as previously described (Manley *et al*., 1999; Maehara *et al*., 2001). To avoid artefacts due to PCR polymerase slippage, a high fidelity DNA polymerase was used and the PCR cycling times were short. To avoid concerns that impurities or other types of contaminants from two different tissue types (blood and testis) could differently affect the PCR performance resulting in misleading findings, samples demonstrating instability were confirmed by concomitant PCR and fragment analysis of a mixed blood and testis paired DNA sample [the results (not shown) indicated the expected finding of multiple peaks]. Additionally, to assure consistency and minimize the possibility of false-negative or false-positive results, several independent repetitive PCR and fragment analysis runs were performed for each sample to rule out artefactual PCR 'stutter' or 'chatter' as a potential source of instability. Thus, the negative or positive instability results were indubitable. Irregularities from the fragment analyser runs were also avoided by assaying blood and

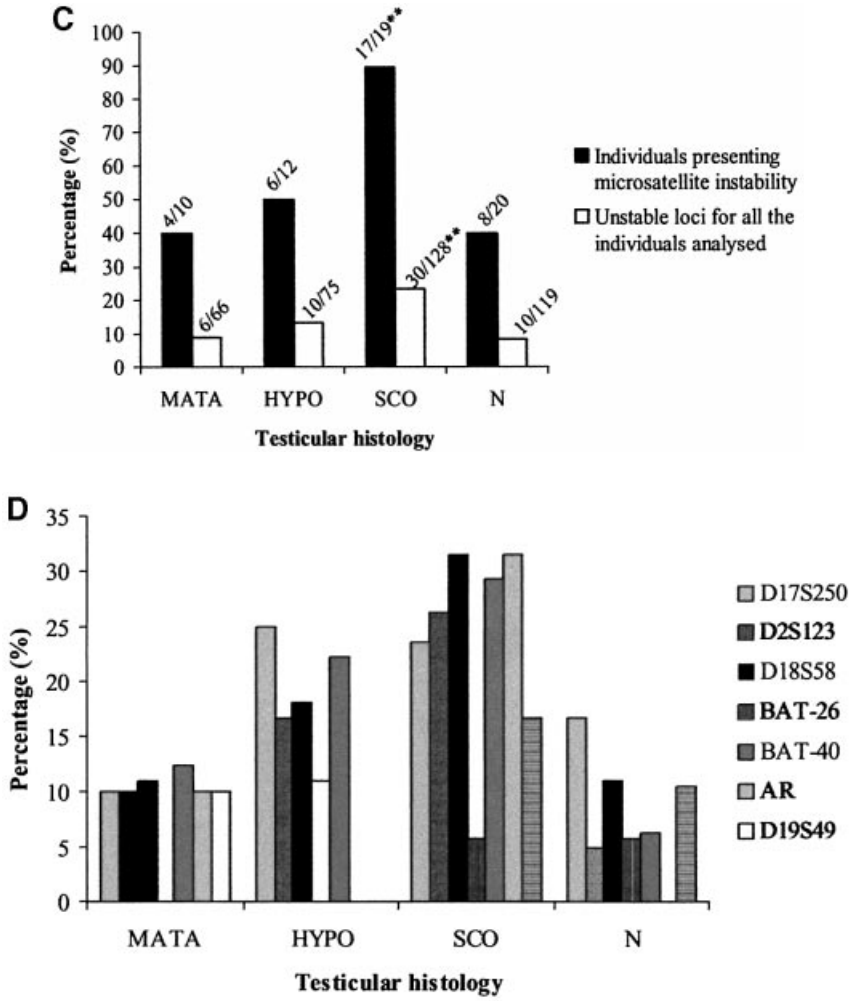


Figure 1. Microsatellite instability in testicular failure patients. Two examples of patients presenting microsatellite stability (**A**) and instability (**B**) at the AR locus. Shown are the GeneScan data tables of the elution times for each PCR fragment and their respective peak heights, with the associated GeneScan histograms showing the *AR* PCR fragment lengths (base pairs; *x*-axis) for the paired blood (upper panels) and testis specimens (lower panels) plotted against the PCR fragment peak height (*y*-axis) after a high-resolution fluorescent electrophoresis run. The peak height is directly proportional to the number of fluorescent fragments detected by the ABI 310 Analyser after capillary electrophoresis. Note the similar (**A**) and different (**B**) migration patterns of the PCR fragment peaks (blue) for blood and testis in comparison to the molecular weight marker (red). In this example of microsatellite instability (**B**), the testis GeneScan histogram peak exhibited a homogeneous shift towards the right, compared with results obtained with the same patient's blood. Thus, all testicular DNA molecules that were amplified and analysed for this patient exhibited an expanded CAG repeat length (with an expansion of two or three repeats, equivalent to 6–9 bp), as compared to the sample of patient's blood DNA. In this particular case, however, the expansion may not have occurred throughout the testis and may simply represent a clonal mutation, as only a small biopsy from one location was available for analysis. Therefore, the results do not guarantee the presence of different cell types in the specimen. In addition, since under-represented alleles may not be amplified with the same efficiency, they may not be equivalently detected despite their presence. Similar results demonstrating instability were obtained for the other loci tested. Microsatellite instability affecting a portion of the cells analysed was also characterized by the appearance of a new peak in the testicular histogram, in addition to the major allele present in the patient's blood histogram. (**C**) Percentage of patients with instability and their respective instability frequency. SCO patients exhibited significantly increased microsatellite instability ($P = 0.007$) and instability frequency ($P = 0.007$), compared with patients with other pathologies and with normal controls (**). No other histological groups presented significant differences when compared with each other or with normal controls. (**D**) Percentage of patients with instability for each of the loci tested. Statistical analysis showed that none of the individual loci presented a significant correlation with any of the testicular pathologies ($P > 0.05$), due to insufficient power. SCO = Sertoli cell-only syndrome; MATA = maturation arrest; HYPO = hypospermatogenesis; N = normal histology.

paired testis samples together. Results demonstrate that microsatellite instability is present in some testicular failure patients at a much higher frequency than in normal fertile men ($P < 0.01$).

SCO men demonstrated both the highest percentage of patients with genomic instability ($P = 0.007$) and the highest instability frequency ($P = 0.007$). In addition, an increased incidence of high microsatellite instability (H-MSI) was also observed in the SCO group. H-MSI, which is considered to be present when >30–40% of the loci analysed show instability, has been proposed to provide a better indication for MMR abnormalities than total microsatellite instability (Tomlinson *et al*., 2002). Although L-MSI (low microsatellite instability) is believed to be a genuine phenomenon that precedes H-MSI, some authors argue that since slippage of any microsatellite can normally occur approximately once in every 1000 cell divisions, if a large enough number of microsatellites are typed, it is likely that differences will be found (Duval and Hamelin, 2002; Tomlinson *et al*., 2002). Although this normal slippage can certainly help explain baseline levels of instability (such as detected in normal controls), L-MSI level differences between controls and the different classes of testicular failure patients were also observed.

In contrast, the maturation arrest and hypospermatogenesis patients did not exhibit significant instability frequency. Surprisingly, maturation arrest patients have been reported to have instability at the D19S49 locus (Nudell *et al*., 2000). A different technical approach and

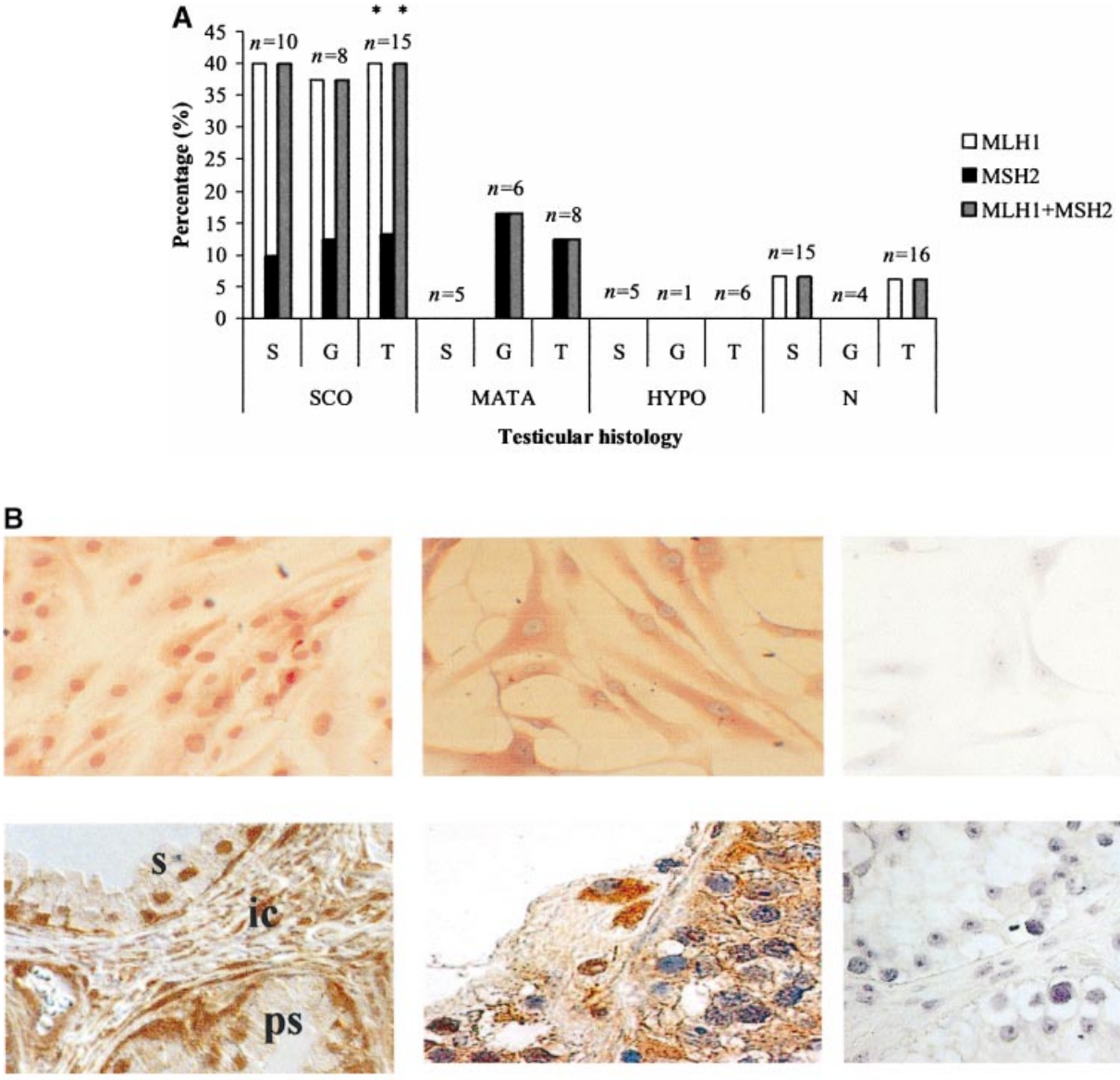


Figure 2. (**A**) Percentage of patients with abnormal DNA mismatch system (MMR) protein expression for each of the different histological categories. Incidence of MLH1, MSH2 and total (MLH1 + MSH2) defects in the testicular somatic (S) and germline (G) cells of each histological group are shown. Total MMR abnormalities (T), corresponding to the combined somatic and germline MMR expression defects, are shown for each protein tested and each pathological category. Total MMR abnormalities, as well as total hMLH1 expression defects, were significantly correlated with the Sertoli cell-only (SCO) group of patients (*$P < 0.05$). (**B**) Expression of DNA MMR proteins in human testis tissues: panels **1**, **2** and **3** (top; left to right) show immunohistochemistry assays performed on fresh, in-vitro cultured testicular fibroblasts; panels **4**, **5** and **6** (bottom; left to right) show immunohistochemistry assays performed on paraffinized testicular sections. All pictures captured with ×200 amplification. (**1**) Infertile patient with normal histology and normal expression of hMSH2. (**2**) SCO patient with abnormal cytoplasmic localization of hMLH1. (**3**) SCO patient with no expression of hMSH2. (**4**) Maturation arrest (MATA) patient with normal expression of hMLH1. Note the positive signal in the nucleus of the Sertoli cells (s), primary spermatocytes (ps) and interstitial cells (ic). (**5**) Abnormal cytoplasmic hMSH2 expression in a MATA patient. (**6**) Absence of hMSH2 expression in a SCO patient.

the small number of patients evaluated by Nudell *et al.* may have influenced the sensitivity of instability detection.

In general, no correlation between the loci affected by instability and the patient's histological characteristics was found ($P > 0.05$). However, hypospermatogenesis patients exhibited a high percentage of instability in the BAT-40 locus (Figure 1d). This may be of importance, as the BAT-40 repeat locus is located within the 3β-hydroxysteroid dehydrogenase (3β-HSD) gene, and the involvement of this protein in testosterone biosynthesis is well-recognized. As differences in the polymorphic repeat lengths within genes influence the protein function, it will be important to understand the role of these polymorphisms in the 3β-HSD gene in hypospermatogenesis.

Similarly, the polyglutamine triplet repeat length within exon 1 of the androgen receptor was one of the loci predominantly affected in the SCO patients (Figure 1d). This *AR* polyglutamine polymorphism is known to influence receptor activity (Mhatre *et al*., 1993; Beilin *et al*., 2000), and this may contribute to the pathology of some SCO syndrome cases. Moreover, *AR* repeat length expansion is associated with some severe diseases that may be inherited by the offspring of these men with microsatellite instability (Casella *et al*., 2001).

Although the complete absence of germ cells in the testis is defined as SCO syndrome, in more than half of these cases patients will show occasional foci of normal spermatogenesis (Silber *et al*., 1995). Therefore, instability may be present in the under-represented germ cell population, and may affect offspring conceived through TESE–ICSI. Importantly, due to the total absence of sperm in the ejaculate of the patients and the small biopsy size, it is impossible to differentiate between the germ and somatic cell lines within these testicular samples with instability. Nevertheless, if only the testicular somatic cell lines exhibit instability, systemic consequences for the patients may still occur. By analogy to cancer patients who exhibit microsatellite instability and to mouse models deficient in MMR, infertility patients with instability may be predisposed to develop tumours (Baker *et al*., 1995; Edelmann *et al*., 1996; Roest *et al*., 1996; Rotman and Shiloh, 1998; Edelmann *et al*., 1999; Lipkin *et al*., 2002). While no history of cancer was reported for this patient population, these patients were young (with the mean age in the lower thirties) and tumours may occur later in life. In fact, impairment of cell proliferation due to MMR defects in an organ with a high rate of cell division (such as testis) would be expected to be apparent sooner than in organs with a lower rate of cell turnover. Conversely, the microsatellite instability and MMR abnormalities identified in testis may not affect other organs—organ and tissue mosaicism occur and play a major role in certain sporadic tumours.

Sertoli cells are not proliferative in adult testis. The differences in repeat length identified in the current study may have occurred through DNA polymerase slippage during development, while these cells were still dividing. Most remarkably, repeat variations might occur in the absence of DNA replication through spontaneous acquisition of secondary alternative structures or through repair attempts after cytotoxic DNA damage (Sinden, 2001). This may explain the presence of genomic instability in the Sertoli cells.

Germ cells may be more sensitive to defects in MMR machinery when compared with somatic cells and these functional defects affect spermatogenesis in animal models (Baker *et al*., 1995; Edelmann *et al*., 1996; Roest *et al*., 1996; Rotman and Shiloh, 1998; Edelmann *et al*., 1999; Lipkin *et al*., 2002). The increased complexity of meiosis compared with the known steps required for mitosis may require more checkpoints for cell cycle progression. Defects in the MMR system might not be enough to impair mitosis, but sufficient to impair meiosis resulting in azoospermia. The fact that the testis exhibits the highest rate of cell proliferation in the human body may also explain the loss of MMR control observed in testicular samples compared with the paired blood samples (which have a lower, although still high, proliferation rate) through MMR system saturation. This may explain why the testicular failure patients did not present with other more severe conditions known to be associated with MMR abnormalities, such as HNPCC.

The immunohistochemistry results support these hypotheses. The incidence of defects in the DNA repair proteins, hMHS2 and hMLH1, was significantly higher in SCO patients when compared with those men with normal spermatogenesis ($P < 0.05$). As expected, hMLH1 defects were the most common ($P < 0.05$). Since these defects were present in both the somatic and mixed (somatic + germinal) cell lines of the patients analysed, SCO individuals may have an enhanced risk for tumour development. Since there is no long-term patient follow-up of the infertile male after diagnosis or treatment (assisted reproductive technology), little is known about the incidence of cancer in the men diagnosed with idiopathic infertility. The occurrence of a malignant tumour will likely be brought to the attention of a physician (other than an infertility specialist), who may not be aware that the patient was infertile. Nevertheless, a relationship between male infertility and testicular cancer has been proposed. In fact, men with an abnormal semen analysis have a 1.6-fold increased risk of developing testicular cancer compared with fertile men (Jacobsen *et al*., 2000).

Unlike the mouse models, hMLH1 defects in the present study were rarely associated with sperm maturation arrest. In fact, hMSH2 defects (not associated with male infertility in the mouse) were observed, mainly in maturation arrest patients. These observations suggest that although evolutionarily conserved, MMR proteins might have slightly divergent functions in rodents and humans. Since some patients with MMR deficiencies presented with some degree of spermatogenesis, some of the proteins analysed may have analogous or redundant functions in the testis.

In conclusion, we report for the first time the presence of significant microsatellite instability in SCO patients. The genomic instability identified does not affect all of the testicular cell subpopulations equally. In addition, defects in the cellular localization and expression of two MMR proteins are demonstrated in SCO patients. These findings provide insight into a new aetiology of SCO syndrome and raise the possibility that there may be consequences for the offspring conceived by ICSI. More studies are needed to definitely prove this suggested association between MMR abnormalities and potential consequences for TESE–ICSI conceived children.

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