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Germline Telomere Attrition is Associated with Human Male Infertility

S. Pathak*, A.S. Multani*, S.H. Sohn, N.J. Gupta***, B.N. Chakravarty*****

Abstract

Meiotic division requires pairing of homologous chromosomes for normal segregation, recombination, and production of haploid gametes. Early in meiotic prophase, chromosomes begin pairing off from the telomeric ends toward the centromere, irrespective of chromosome morphology. Since synaptonemal complex formation during zygotene/early pachytene begins at the telomeres, we hypothesized that in some infertile human males telomeric chromosomal DNA may be constitutionally reduced and, therefore, insufficient to initiate homologous synapsis, resulting in asynaptic meiosis and azoospermic infertility. To test this hypothesis, we measured and compared percent telomeric DNA in peripheral blood lymphocytes (PBLs) and testicular biopsies from infertile males (cases) and age-matched normal fertile males (controls). Quantitative fluorescence in situ hybridization (Q-FISH) revealed that cases had significantly less telomeric DNA than did controls ($P < 0.001$; $P = 0.002$ respectively). In addition, in experiments with mouse testes exposed to the telomerase-inhibiting and sterility-inducing drug, cytarabine *in vivo,* silver staining and analysis of synaptonemal complexes showed that homologous chromosome pairing was prevented due to the loss of telomeric DNA. Taken together, these observations indicate that constitutionally reduced amounts of telomeric DNA in somatic and germ cells may be a crucial determinant in human male sterility.

Keywords: Homologous Pairing; Infertility; Pachytene; Synaptonemal Complex; Telomeric DNA.

Introduction

The single most important modification of mitosis is meiosis, which gives rise to the haploid gametes, sperm in male and ovum in female. During meiosis, chromosome number is reduced to half because the cell nucleus divides twice while the chromosomes replicate only once. Accurate segregation and reduction of chromosome number requires that the homologous chromosomes or segments of chromosomes pair off during the early stages of meiotic prophase (Pathak and Hsu, 1979; von Wettstein *et al*., 1984). Synapsis, or pairing, of chromosomes is a prerequisite for homologous recombination, being initiated at the telomeric ends and proceeding toward the centromere, irrespective of chromosome morphology (Pathak, 1983; Pathak and Hsu, 1976; Pathak and Lin, 1981; Dernburg *et al*., 1995; Zickler and Kleckner, 1998). In acrocentric chromosomes, pairing starts from the telomeric ends and proceeds towards the centromere, whereas in metacentric chromosomes synapsis can be bidirectional, starting from both termini and proceeding towards the centromere (Pathak and Hsu, 1976 and 1979; Pathak *et al*., 1980; Elder and Pathak, 1980; Ishikawa and Naito, 1999). However, exceptions to this rule have been reported, especially in X and Y chromosome pairing and segregation (Pathak, 1983; Pathak and Hsu, 1976; Pathak *et al*., 1980).

Telomeres, which are present at the termini of eukaryotic chromosomes, consist of TTAGGGn sequences that are conserved in all vertebrates (Moyzis *et al*., 1988; Meyne *et al*., 1990). Functional telomeres of linear chromosomes are associated with

Author's Affiliation: *Departments of Genetics, The University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030, USA. **Department of Animal Science and Biotechnology, Chinju National University, Chinju 660-758, Republic of Korea. ***Institute of Reproductive Medicine, HB-36/A/3 Sector 3, Salt Lake, Kolkata-700097, India.

Corresponding Author: S. Pathak, Department of Genetics, Unit 1010, M. D. Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, Texas 77030, USA.
E-mail: spathak@mdanderson.org

telomeric repeat binding factor (TRF) and other proteins (Scherthan *et al*., 2000). Human telomeres are also associated with the nuclear matrix (de Lange, 1992). This association may have several purposes: (a) to determine the domain of individual chromosomes within the nucleus; (b) to prevent the chromosomal arms from entangling; (c) to help search for homologous partners; (d) to initiate pairing of homologous chromosomes during meiotic prophase; and (e) to protect chromosomal integrity by absorbing insults from external challenges that may enter the cell nucleus. During each cell division, a portion of the telomere is lost as a result of the end-replication problem (Watson, 1972, Olounikov, 1973). The telomeres, therefore, become progressively shorter and leads to senescence cells both *in vivo* and *in vitro* (Harley *et al*., 1990; Hastie *et al*, 1990; Greider, 1996). The male germ cells, however, appear to be an exception to this process because of the presence of the enzyme telomerase, which adds on nucleotides at the chromosomal termini (Allsopp *et al*., 1992; Kim *et al*., 1994; Blackburn and Greider, 1995; Banerjee *et al*., 1998, Kalmbach *et al*., 2013). Since the pairing of homologous chromosomes starts from the telomeric ends, we hypothesized that those males who have developed "normally," but are infertile, might have telomere dysfunction or have constitutionally reduced amounts of telomeric DNA in their genomes (Pathak *et al*., unpublished data; Dawe *et al*., 1994; Multani et al., 2002).

To test this hypothesis, we analyzed and compared telomere dynamics in the peripheral blood lymphocytes (PBLs) and testicular biopsies of infertile human males (cases) and age-matched healthy fertile males (controls). In addition, we studied the effect of telomeric DNA reduction on the normal meiotic pairing of homologous chromosomes in male mice injected with 1-beta-D-arabinofuranosyl-cytosine (cytarabine, ara-C). Cytarabine, a chemotherapeutic drug very effective against acute myeloid leukemia (Keating *et al*., 1982; Champlain and Gale, 1987; Grant, 1998), is known to induce telomere attrition in cancer cells *in vitro* (Multani *et al*., 2000) and mild to severe sterility in cancer patients treated with it (Lendon *et al*., 1978; Wallace *et al*., 1991).

Materials and Methods

Human Subjects

Seventeen adult males with infertility caused by azoospermia (cases) and 11 normal age-matched fertile males (controls) were enrolled in the present study. Most testicular biopsies and PBL samples were collected at the Institute of Reproductive Medicine (Kolkata, India) and, after fixation, shipped to The University of Texas M.D. Anderson Cancer Center, Houston, TX, for further analysis. In each case, a written consent was obtained from the patient and control subject before sample collection.

PBL Culture and Cytological Preparations

Peripheral blood (10-12 mL) was collected from each male subject (13 infertile patients and 9 fertile controls), and stored in heparinized tubes. Whole blood culture was initiated by adding 1 ml of blood to 9 ml of RPMI 1640 medium rich in folic acid supplemented with 20% fetal bovine serum. All cultures were incubated for 72 h at 37^0C and then harvested following the standard air-drying technique (Pathak, 1976). Acetic acid-methanol- fixed lymphocytes were shipped to Houston from Kolkata, India, for further analysis. Fixed samples of PBLs from both patients and healthy controls were coded at the time of their analyses.

Forty to 50 Giemsa- (G)- banded metaphases from each case and control were evaluated for chromosomal anomalies, especially for marker chromosomes and for the incidence of telomeric associations (TAs).

Human Testicular Biopsy Preparations

Testicular biopsy specimens obtained from 4 infertile and 2 fertile males were processed for cytologic examination according to the standard meiotic chromosome preparation techniques as described previously (Pathak and Hsu, 1979).

Cytarabine Injection in Male Mice and Cytological Preparation

Cytarabine (Sigma; St. Louis, MO) was mixed with phosphate-buffered saline (PBS) to make stock solution. Then, each testis of 7 Swiss male mice was injected with 2.0 mg of the cytarabine in stock solution. Three other Swiss male mice were injected with PBS only, as control. Cytarabine-injected and control mice were sacrificed for air-dried meiotic preparations at 5 and 16 h and 1, 2, 5, and 7 days after drug exposure. Air-dried meiotic preparations were made and stained with either Giemsa or Ag-NOR staining procedure for synaptonemal complex analysis by light microscopy (Pathak and Hsu, 1979).

Fluorescence in Situ Hybridization Analysis of Telomeric DNA

Cytological preparations of human PBLs and testicular biopsies were processed and subjected to fluorescence in situ hybridization (FISH) analysis of telomeric DNA according to a standard technique (Multani *et al.*, 2000). For this, CY-3-conjugated peptide nucleic acid (PNA) telomeric probe (DAKO Corporation, Carpinteria, CA), was used, according to the manufacturer's protocol but with slight modifications (Multani *et al.*, 2000). The telomeric signals in interphase nuclei were quantified by a Metaview image analysis system version 3.6a (Universal Imaging Co., Westchester, PA). Percent telomeric area, defined as the total telomeric DNA length in a given nucleus expressed as percent of total nuclear area, was determined. Fifty to 100 nuclei per sample were evaluated to determine the mean percent telomeric DNA.

Statistical Analysis

All data were analyzed using SPSS (version 10.0) software (SPSS, Chicago, IL) and subjected to factorial and hierarchical analysis of variance.

Results

Clinical Characteristics of Patients

The clinical features of the patients, including age, levels of lutinizing hormone (LH), follicle-stimulating hormone (FSH), karyotypes, and percent telomeric DNA, are shown in Table 1. Data on the levels of different hormones were not available for controls. The levels of LH and FSH in the cases tested were within normal ranges. Cases ranged in age from 29 to 50 years (median, 36 year). Controls ranged in age from 30 to 43 years (median, 36 year).

Table 1: Clinical features and percent telomeric DNA of fertile and infertile males*

Subject	Age (years)	LH (1.5-9.3 mIU/mL)	FSH (1.4-18 mIU/mL)	Karyotype	% Telomeric DNA ± SE
Controls (PBLs)					
1. HB2256	35	-	-	46,XY	1.84 ± 0.13
2. HB2255	30	-	-	46,XY	1.55 ± 0.13
3. HB2251	29	-	-	46,XY	1.81 ± 0.12
4. HB2165	46	-	-	46,XY	2.42 ± 0.18
5. HB2166	33	-	-	46,XY	2.40 ± 0.20
6. HB2282	36	-	-	46,XY	1.39 ± 0.12
7. HB1598	34	-	-	46,XY	1.50 ± 0.12
8. HB2346	50	-	-	46,XY	1.68 ± 0.14
9. HB2258	40	-	-	46,XY	1.25 ± 0.10
Patients (PBLs)					
1. HB2220	37	1.5	8.4	46,XY	0.60 ± 0.06
2. HB2225	40	0.5	3.2	46,XY	0.62 ± 0.07
3. HB2227	40	5.6	11.8	46,XY	0.59 ± 0.06
4. HB2228	34	3.9	13.2	46,XY	0.78 ± 0.07
5. HB2234	33	8.6	10.0	46,XY	0.66 ± 0.06
6. HB2235	42	5.1	6.1	46,XY	0.78 ± 0.07
7. HB2345	43	6.3	4.1	46,XY	1.21 ± 0.07
8. HB2350	31	2.8	5.1	46,XY	0.86 ± 0.09
9. HB2222	30	3.7	5.5	46,XY	0.81 ± 0.97
10. HB2223	37	4.9	2.3	46,XY	0.73 ± 0.05
11. HB2224	33	2.6	8.6	46,XY	0.96 ± 0.06
12. HB2226	40	0.8	3.8	46,XY	0.46 ± 0.04
13. HB2231	43	10.4	14.6	46,XY	0.70 ± 0.13
Controls Testicular Biopsy					
1. SP5146	36	1.9	3.6	46,XY	2.00 ± 0.21
2. SP5153	29	-	-	46,XY	1.66 ± 0.09
Patients Testicular Biopsy					
1. SP5149	31	9.6	34.4	46,XY	0.78 ± 0.21
2. SP5150	39	8.3	43.3	46,XY	0.93 ± 0.10
3. SP5151	33	7.0	24.6	46,XY	1.04 ± 0.07
4. SP5152	37	7.1	34.8	46,XY	1.16 ± 0.09

LH = luteinizing hormone; FSH = follicle-stimulating hormone; PBLs = peripheral blood lymphocytes; SE = standard error of the mean.

Unfortunately, PBL samples were not available from all cases and controls whose testicular biopsies were studied, nor were testicular biopsies available from all cases and controls whose PBLs were studied. All samples analyzed showed the normal 46, XY chromosome constitution. The frequency of TAs (telomeric associations) in metaphase from cases were slightly elevated compared to controls (data not shown).

Q-FISH Analysis of PBLs and Testicular Cells

The results of Q-FISH analysis of PHA-stimulated PBLs and unstimulated testicular cells from cases and controls are shown in Table 1. Representative preparations of PBLs and testicular cells are shown in Figure 1. The mean percent telomeric DNA was significantly lower for PBLs from cases than for PBLs from controls (0.75% vs. 1.76%, *P<0.001*). The same was true for testicular cells (1.00% vs. 1.81%, *P=0.002*). From this comparison, it was inferred that the percent telomeric DNA in the germ (testicular) cells was also significantly lower for cases than for controls.

Synaptonemal Complex Formation in Mouse Testes Injected with Cytarabine

Figure 2 shows silver-stained synaptonemal complex preparations made from cytarbine-injected and PBS-injected (control) testes. In the control preparations, the synaptonemal complexes were morphologically normal (Figures 2A and B), and bivalents (19 autosomal and the X-Y sex bivalents) were easily counted in all pachytene stages. As shown in Figure 2A, synapsis formed along the entire length of the linear homologous chromosomes. However, as shown in Figure 2B, the lateral elements in many of the bivalents were in the process of pairing off. The situation in the testes of mice injected with cytarabine was just the opposite in many zygotene/ pachytene spermatocytes. As shown in Figure 2C, the lateral elements of the two homologous chromosomes were far apart without any sign of synapsis. This condition was observed in almost 90% of the zygotene/ pachytene stages. Even in Giemsa-stained preparations, the two homologous chromosomes were rarely completely paired (data not shown). We have used the term early diplotene-like morphology (EDM) to describe such configurations. The frequency of EDM in the testes harvested after various intervals of drug injection is shown in Table 2. The frequency of EDM was highest (72%) 16 h after injection, then began declining until reaching its lowest value (31.7%) on day 7. This value was very similar to that for the control testis (30.6%). In addition, different degrees of chromosome breakage, TAs and translocations characteristics of meiotic dysfunction were seen at diplotene, diakinesis, and metaphase stages I and II (data not shown).

Table 2: Frequency of early diplotene-like morphology (EDM) in mouse testes treated with cytarabine (2.0 mg/testis)

Time	Total no. of pachytenes examined	No. of pachytenes with EDM	% EDM
2 hr	200	66	33.0
5 hr	279	179	64.1
16 hr	169	122	72.1
1 days	227	144	63.4
2 days	181	76	41.9
5 days	214	102	47.6
7 days	63	20	31.7
Control	173	53	30.6

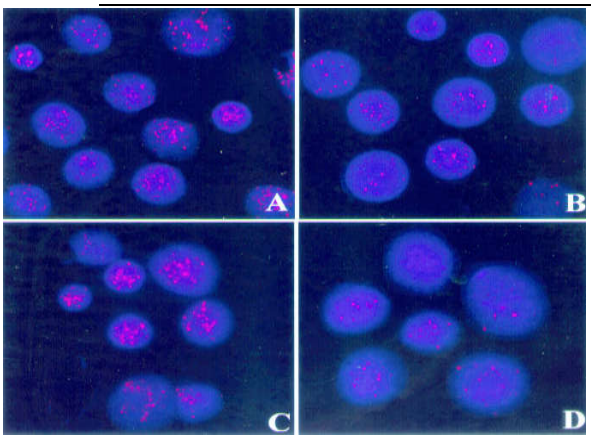

Fig. 1: FISH preparations of peripheral blood lymphocytes and testicular cells from infertile and normal fertile human males showing telomeric DNA signals in interphase nuclei. Nuclear DNA was stained with DAPI (blue); telomeric DNA was stained with CY-3-labeled anti-telomeric PNA (red). Here, lymphocytes from a fertile male **(A)** have significantly more telomeric DNA than do those from an infertile male **(B),** and testicular nuclei from a fertile male **(C)** have relatively more telomeric DNA than do those from an infertile male **(D).** All microphotographs were taken at the same magnification.

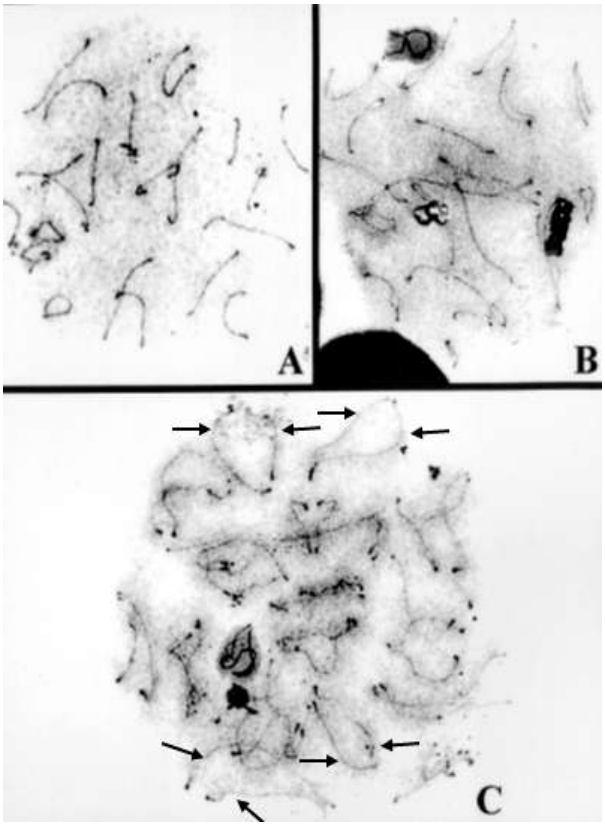

Fig. 2: Silver-stained representative pachytene spermatocytes from cytarabine-injected and PBS-injected (control) male mice showing pairing of the homologous chromosomes and synaptonemal complex formation. **(A, B)** Complete or almost complete pairing of homologous chromosomes (19 autosomal and the X-Y sex bivalents) in testis from a normal mouse. **(C)** Incomplete formation of the synaptonemal complexes in testis from a cytarabine-injected mouse in which two lateral elements can be clearly seen (arrows). Interestingly, drug-treated testes showed asynaptic meiosis with fragmented bivalents in diakinesis and metaphase I (data not shown).

Discussion

Meiosis is a special form of cell division that occurs in the testes and ovaries. Its products are haploid gametes (sperm and ovum), the connecting links between generations. Mitosis, meiosis, and spermiogenesis constitute spermatogenesis. Testicular stem cells divide and differentiate into spermatocytes, spermatids, and spermatozoa. During these processes, it is vital that the optimal amount of telomeric DNA be maintained. Even a slight reduction in telomeric DNA may impair homologous chromosome pairing, resulting in asynaptic meiosis and, ultimately, sterility. Failure of chromosomal synapsis in mice lacking Dmc1 results in meiotic prophase arrest (Pittman *et al*., 1998, Thilagavathi *et al*., 2013). Unlike most human somatic cells, testicular cells have a telomerase activity that maintains optimal telomere length in order to ensure proper alignment during synapsis (Wright *et al*., 1996). Telomerase activity does not appear to be age-specific since it has been reported in human testes of different ages (Kim *et al*., 1994; Burger *et al*., 1997). Telomerase is involved in the elongation of telomere and thus helps germ cells to divide continuously (Counter, 1996; Greider, 1996). In a detailed analysis of telomerase activity in the testes of infertile male rats, Fujisawa and associates (1998) observed telomerase activity in the pachytene and round spermatids but not in spermatozoa. Others have noted that telomerase activity is higher in spermatids than in pachytene spermatocytes (Eisenhaur *et al*., 1997).

In the present study, we determined the amount of telomeric DNA in the PBLs and testes of selected azoospermic human males and age-matched fertile controls. Since the only target for telomerase is the telomere, we decided not to measure the activity of telomerase in our case and control samples. Our data (Table 1) indicate that infertile males have a significantly lower percentage of telomeric DNA in their PBLs than do age-matched fertile controls. The 95 % confidence interval for the testes controls and cases (patients) were 1.639 – 1.972 and 0.879 – 1.127, respectively. The same for the PBL group was 1.684 – 1.830 and 0.688 – 0.815. The significance between the controls and patients telomeric DNA % within the PBLs is $P < 0.001$, and the same for the testes group is $P = 0.002$.

This implies in turn that the genomes of infertile patients contain markedly less telomeric DNA than do those of controls, irrespective of the tissue tested (testes vs. PBL). In fact, since our results in PBLs and testes were so similar, we conclude that the blood test alone would be a good surrogate for the testicular telomeric DNA measurement. Our hierarchical stastistical analysis of the present data supported this conclusion. A similar observation has been reported earlier while comparing the telomere length in human sperms and leukocytes (Aston *et al*., 2012).

Previously, we have reported that attrition of telomeric DNA is the first chromatin-based alteration that occurs in cells undergoing programmed cell death (Pathak *et al*., 1994 and 1998; Multani *et al*., 2000). We have also reported constitutional germline attrition of telomeric DNA in subgroups of human populations. Such individuals are either predisposed to various types of malignancies or have reproductive problems (Pathak *et al*., unpublished data; Multani *et al*., 2002, Calado and Young, 2009). In light of these observations, we hypothesized that some infertile human males may have germline attrition of their telomeric DNA. This hypothesis is supported by the present data showing that azoospermic males had

significantly lower amounts of telomeric DNA (0.75% ± 0.03) in their PBLs than did controls (1.76% ± 0.04).

One of the important functions of telomeres is to determine the domain of individual chromosomes within the cell nucleus. We have previously reported that pairing of homologous chromosomes during meiosis starts from the telomeric ends and proceeds toward the centromere irrespective of chromosome morphology (Pathak and Hsu, 1976 and 1979). A similar observation has been made in the budding yeast (Rockmill and Roeder, 1998). In other words, telomere length plays an important role in the initial stage of synaptonemal complex formation (Pathak and Hsu, 1976; Elder and Pathak, 1980; Pathak and Lin, 1981; von Wettstein *et al.,* 1984). Short telomeres may be rendered inactive, resulting in an asynaptic type of spermatogenesis and ultimately azoospermia or infertility. Individuals with reduced amounts of telomeric DNA in their somatic cell chromosomes may show genetic instability, double strand DNA breakage, aberrant DNA repairs and predisposition to certain epithelial malignancies (Sahin and DePinho, 2010). In fact, two of our infertile male patients in India have developed prostate cancer (unpublished data), a disease that is much less common in India than it is in the United States.

Unlike human somatic cells, normal mouse somatic cells have telomerase activity. However, studies in telomerase knockout mice (mTR-/-) have clearly shown how important telomerase is in telomere maintenance and viability (Blasco *et al*., 1997; Lee *et al*., 1998). Earlier reports have also shown that mice with genetically altered mTR components and dysfunctional telomeres are less fertile, show chromosomal instability, and are predisposed to certain epithelial malignancies that do not occur spontaneously in wild-type animals (Herrera *et al.,* 1999; Rudolph *et al*., 1999; Hande *et al., 1999;* Artandi *et al.,* 2000, Chang et al., 2004). This mouse model clearly supports our present observations in infertile human males, who may be predisposed to premature aging and development of certain epithelial malignancies, and warrant further studied in humans to test our hypothesis.

The results of our experiments in cytarabine-injected male mice clearly demonstrate that loss of telomeric DNA impairs homologous pairing in meiosis (Figure 2). Therefore, they support a number of recent findings by us and others. We have earlier demonstrated that human and murine cancer cells treated with cytarabine *in vitro* lose telomeric DNA, which is cleaved and then extruded from the cell (Multani *et al*., 2000). Hemann and associates (2001) have shown that telomere dysfunction triggers apoptosis in testicular cells, but that chromosome asynapsis may not be the cause of infertility in mice. Also, Liu *et al.,* (2002) have reported that telomeric dysfunction may eventually disrupt meiotic spindles and cause misalignment of chromosomes during meiotic division of oocytes in late-generation (G4) TR -/- mice. Taken together, these observations support our hypothesis that attrition of telomeric DNA is associated with infertility and also be a predisposing factor for certain solid tumors in some human males.

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