Chromosome Research (2006) 14:527–534
DOI: 10.1007/s10577-006-1050-9

Sporadic aneuploidy in PHA-stimulated lymphocytes of Turner's syndrome patients

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Received 28 August 2005. Received in revised form and accepted for publication by Mike Goldman 22 February 2006

Key words: autosomal aneuploidy, fluorescence *in-situ* hybridization, mosaicism, Turner's syndrome

Abstract

In line with the view that aneuploidy destabilizes the karyotype, initiating an autocatalytic process that gives rise to further loss and/or gain of chromosomes, we examined whether a constitutional aneuploidy such as monosomy for one chromosome is associated with sporadic loss and/or gain of other chromosomes. We used PHA-stimulated lymphocytes from eight women with Turner's syndrome (six displayed X chromosome monosomy ranging from 60.2% to 97.9%, and two were below 10%), and eight healthy women who served as a control group. Fluorescence *in-situ* hybridization (FISH), applied at interphase, was used to evaluate the level of aneuploidy for three randomly selected chromosomes (autosomes 8, 15 and 18) in each sample. For each tested chromosome, our results showed a significantly higher level of aneuploid cells in the samples from patients than in those from controls ($p < 0.01$). The mean level of aneuploid cells for all three tested autosomes was almost twice as high in the patient samples as in the control samples ($p < 0.002$). It is noteworthy that, in the Turner's syndrome patients, X chromosome disomic cells also displayed increased levels of aneuploidy. It is possible that monosomy of X chromosome in female cells destabilizes their own genome and also affects X disomic cells in the region. One may also speculate that a common factor(s) is involved with both constitutional and sporadic aneuploidy.

Introduction

Turner's syndrome is a unique viable human chromosome monosomy (Hassold *et al*. 1992, Lora-Sanchez *et al*. 1992); it is associated with numerous abnormalities, including congenital defects in various systems, which are not restricted to pure monosomy of X chromosome (Zinn *et al*. 1993). Recent efforts to uncover the genetic complexity of Turner's syndrome have focused on the search for the critical DNA regions on chromosome X which, when missing, give rise to the syndrome. Since a large portion of X-chromosome genes on one X homologue (for male dosage compensation) is inactivated in eutherian female cells, the main focus was attributed to genes escaping X-chromosome inactivation (Carrel *et al*. 1999), but the findings were not definitive. Recent information on the inactivation state of X-linked genes shows that almost 40% of genes that escape X-inactivation display great variation in expression in various inactive X chromosomes (Carrel & Willard 2005). This throws into question the view that the

reduced product-amount of genes escaping X-inactivation is critical and as such is involved in dictating the Turner phenotype.

Other authors who attempted to explain the large number of congenital and acquired abnormalities in patients with aneuploidy conditions, such as Down's syndrome, suggested that the aneuploidy status (the abnormal number and balance of chromosomes) is itself the machinery causing the associated phenotypic defects (Shapiro 1983). This is in line with the view that aneuploidy causes genes present on the genome in normal dosage to go out of control (Amiel *et al.* 1998, 1999, Reish *et al.* 2002). If this is indeed the case, then the missing products of the X chromosome in Turner's syndrome disrupt the obligatory integration of the entire genome, affecting also genes residing on chromosomes which are presented in the monosomic genome in normal dosage – two copies each. Indeed, it was recently shown that genes such as *CMYC*, *TP53* and *AML1*, located on autosomes 8, 17 and 21, respectively, showed alterations of DNA replication properties thought to be associated with transcription capability and/or methylation capacity when present in cells of Turner's syndrome patients (Reish *et al.* 2002).

This accords with the idea that, because of the changes in large batteries of genes, an unbalanced chromosome complement strongly affects gene products: it causes malfunction of various cellular pathways, including DNA replication and chromosomal segregation (Antonarakis *et al.* 2004), giving rise to continuous changes in the karyotype that are reflected in new abnormal chromosome complements (Duesberg & Rasnick 2000, Rasnick 2002, Duesberg & Li 2003).

We investigated whether monosomy of X chromosome in Turner's syndrome genotypes is associated with non-chromosome-specific aneuploidy. Our results show that monosomy of an X chromosome is coupled with sporadic losses and gains of autosomal chromosomes.

Materials and methods

Subjects

The study included eight females diagnosed with Turner's syndrome, designated T1–T8, and eight control females, designated C1–C8. The patients ranged in age from 1 to 39 years, mean 16.3 ± 13.7 years. The age-matched control subjects were healthy and had a normal karyotype based on G-banded metaphase spreads.

Cell culture

Each subject donated 5 ml of peripheral blood, and cell cultures of PHA-stimulated lymphocytes were set up according to a standard protocol (Reish *et al.* 2002). Following harvesting, cells were fixed and stored in suspension in the fixative solution at −20°C until used for FISH analysis.

Probes

Four directly labeled chromosome-specific probes (Vysis, USA) were used: (1) for chromosome 8 (320008; CEN 8), (2) for chromosome 15 (320015; CEN 15), (3) for chromosome 18 (320018; CEN 18), and (4) for chromosome X (320000; CEN X).

In-situ hybridization

Two-color FISH was performed. Each of the autosomal probes was mixed with the X probe, and the probe solution was prepared according to the standard protocol as recommended by Vysis (USA), with a few minor changes. Cells were dropped onto two well slides (Insitus Biotechnologies, USA) without any pretreatment. Five microliters of the mixed probe solution was placed on the targeted area of the sample slides and covered with a 12 mm round silianized cover slip (Insitus Biotechnologies, USA) and sealed with rubber cement. The slides were placed in a micro-heating system (Hybrite; Vysis, USA), and subjected to 6 min denaturation at 76°C and 18 h hybridization at 37°C.

Post-hybridization treatment

Post-hybridization washes consisted of immersing the slides for 2 min in a solution of 0.4 × SSC, pH 7.0, with 0.3% NP40, at 72°C, followed by 2 min in 2 × SSC with 0.1% NP40 at room temperature in a shaking water bath. After brief drying, the slides were covered with antifade containing 4,6-diamidino-2-phenylindole (DAPI; 3 μg/ml; Vector Laboratories, USA), covered and stored at −20°C until analyzed.

Cytogenetic evaluation

Slides were analyzed blindly on an Olympus BH2 fluorescent microscope, using a triple bandpass filter (Chroma Technology, Brattleboro, VT, USA).

Three or four independent hybridization procedures were performed with the CEN X probe to detect the frequency of 45,X cells in the samples of the Turner's syndrome patients and the controls. Between 194 and 205 cells were scored from each hybridization, and the frequency of 45,X cells in each sample was the mean of the three or four hybridizations performed (Figure 1).

The level of aneuploidy in autosomes 8, 15, 18 was assessed by screening about 200 cells for each autosome using two-color FISH with the probe for the autosome in question and the X chromosome probe. The number (one, two or three) of signals of the autosome in question and the X chromosome were recorded from each cell. All samples were evaluated by two independent cytogeneticists.

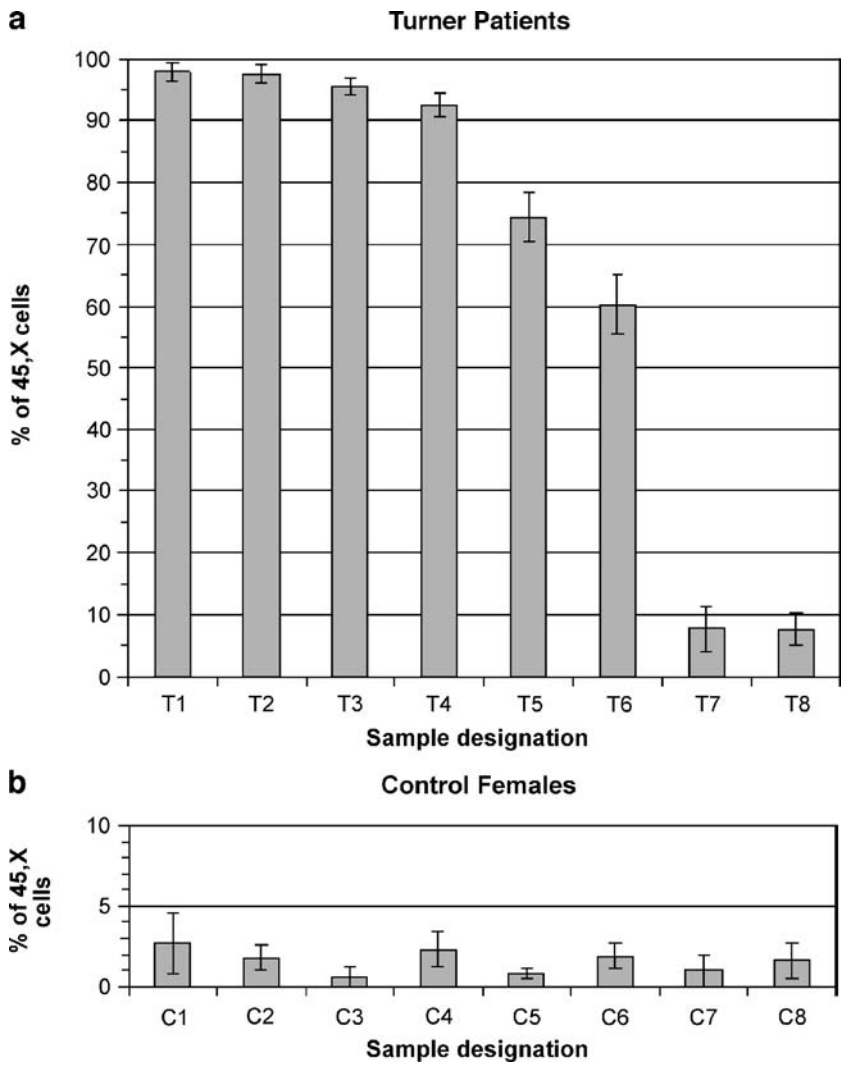

Figure 1. The means and standard deviations of the frequency (%) of 45,X cells in samples of (**a**) Turner's syndrome patients (samples T1–T8) and (**b**) control females (samples C1–C8). Each value is the mean of three or four independent hybridization procedures with the X-chromosome-specific probe, with at least 200 interphase cells screened for each sample following a single hybridization. Thus, each value of each sample is based on at least 600 cells.

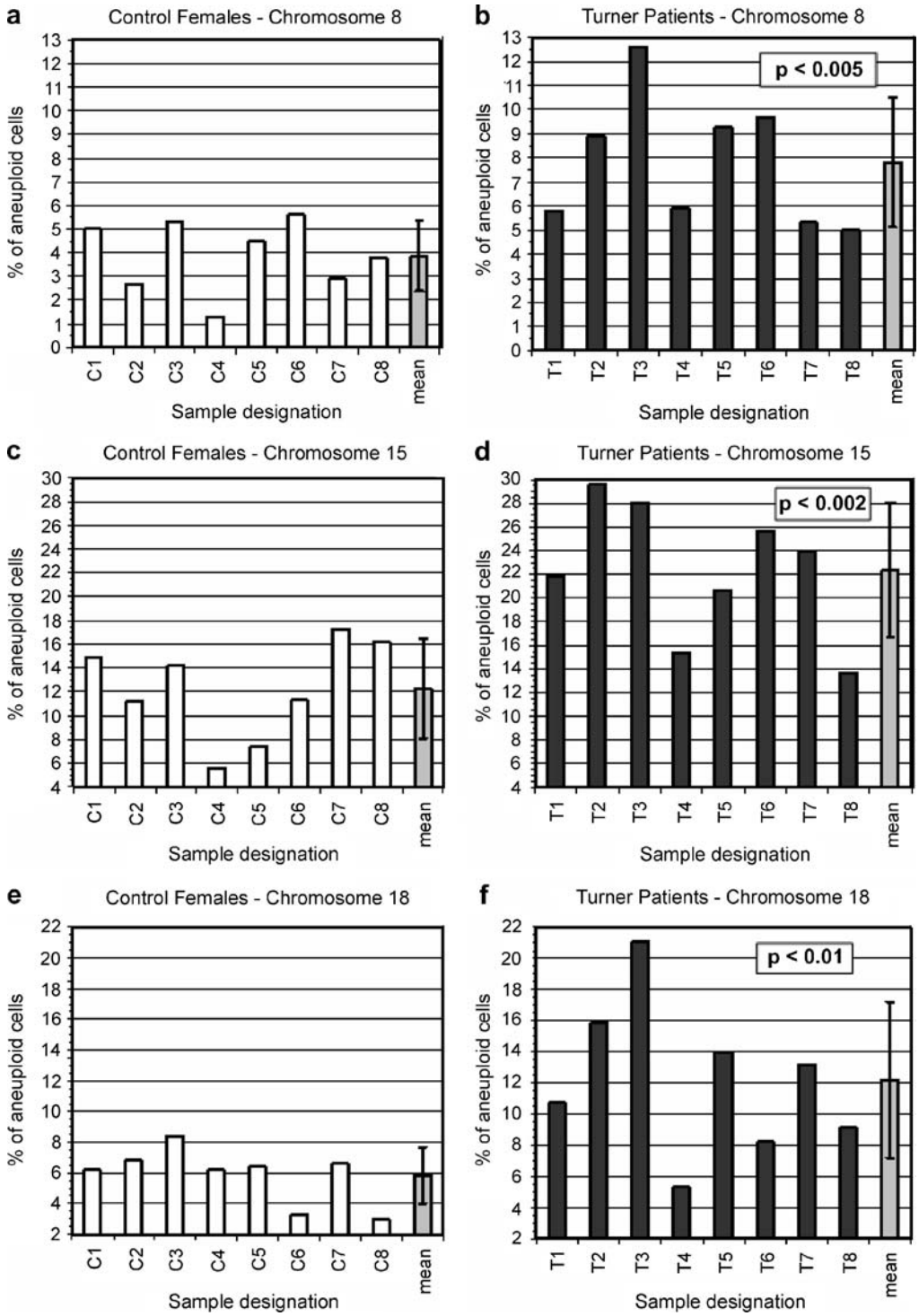

Figure 2. The frequency (%) of cells exhibiting aneuploidy (losses plus gains) for chromosome 8 (frames **a** and **b**), chromosome 15 (frames **c** and **d**), and chromosome 18 (frames **e** and **f**) in samples of control subjects (samples C1–C8) and Turner's syndrome patients (samples T1–T8). The last bars in each frame denote the mean and standard deviation of the group of samples in the frame. For each chromosome at least 200 interphase cells for each sample were screened. *P* = the level of significance.

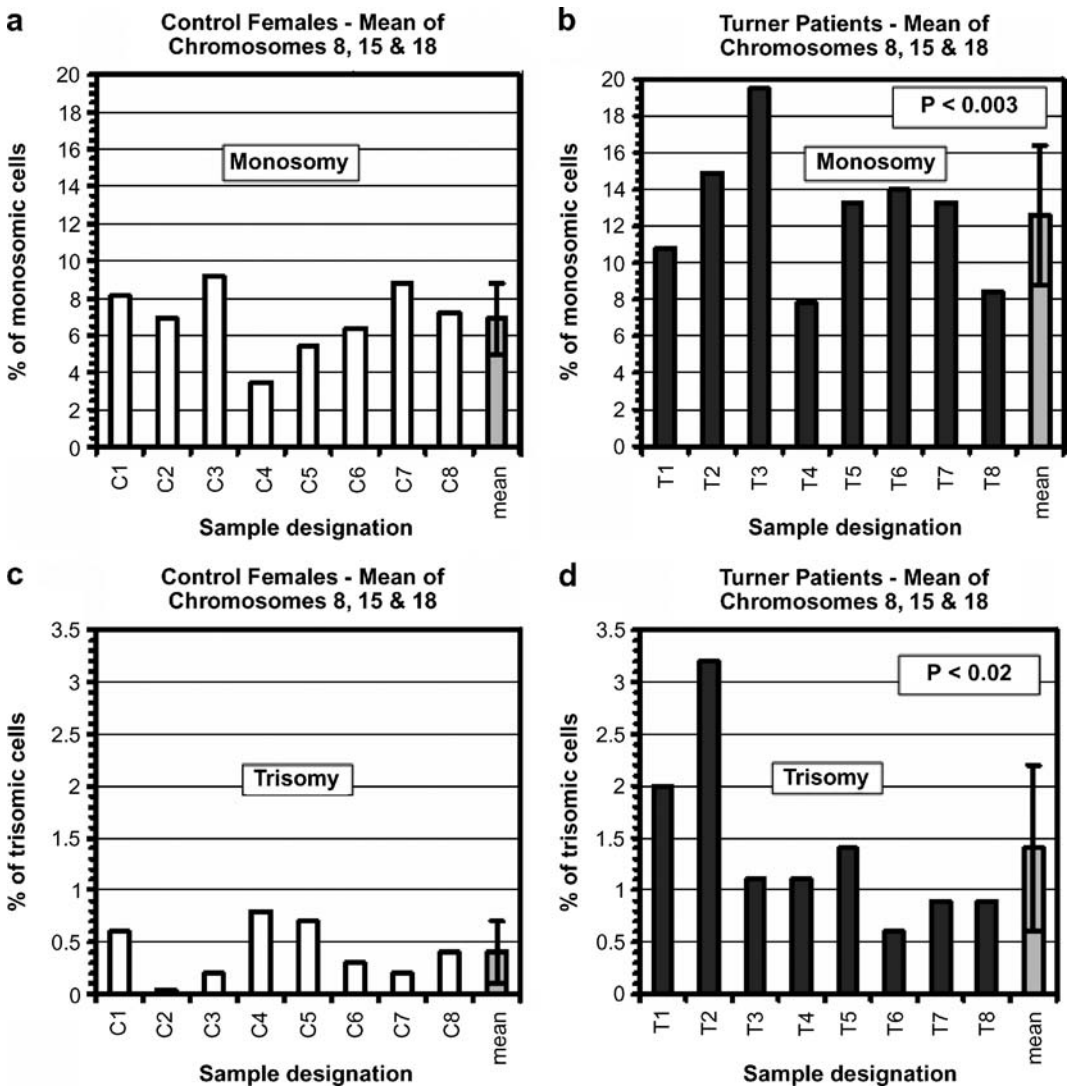

Figure 3. The mean frequency (%) of cells exhibiting losses (monosomy; frames **a** and **b**) and gains (trisomy; frames **c** and **d**) for chromosomes 8, 15 and 18 in samples of control subjects (samples C1–C8) and Turner's syndrome patients (samples T1–T8). The last bars in each frame denote the mean and standard deviation of the group of samples in the frame. Each value is based on at least 600 interphase cells. *P* = the level of significance.

Results

The Turner's syndrome samples showed a large variation in the frequency of X-chromosome monosomy, ranging from 7.7 ± 1.8% to 97.9 ± 1.4% (Figure 1a), compared to only a few 45,X cells, ranging from 0.6 ± 0.6% to 2.7 ± 1.9%, in the control samples. This indicated a high hybridization affinity (above 95%) of the X-chromosome specific probe (Figure 1b).

The application of FISH at interphase with chromosome-specific probes for chromosomes 8, 15 and 18 (randomly chosen) in the same blood cell preparations as above showed that the frequencies of aneuploid cells in these chromosomes of the Turner

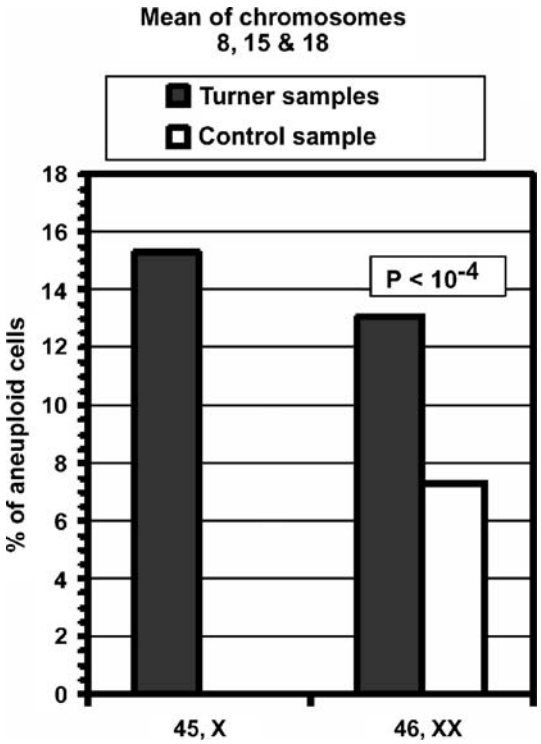

Figure 4. The mean frequency (%) of cells exhibiting aneuploidy (losses plus gains) in populations of 45,X and 46,XX cells derived from Turner's syndrome patients, and in the total population of cells derived from control subjects. Each bar denotes the values of aneuploidy in pooled cell populations of all samples within the designated group. Note that the 46,XX cell population derived from Turner's syndrome patients also displayed a significantly higher level of aneuploidy ($p < 10^{-4}$) than that observed in the cell population derived from control subjects.

samples were almost two-fold higher than those of the control samples, which all exhibited relatively low frequencies of aneuploid cells (Figure 2). The corresponding means for patients and controls were 7.8 ± 2.7% vs 3.9 ± 1.5% for chromosome 8 (Figure 2a and b; $p < 0.005$), 22.4 ± 5.7% vs 12.3 ± 4.2% for chromosome 15 (Figure 2c and d; $p < 0.002$) and 12.2 ± 5.0% vs 5.9 ± 1.8% for chromosome 18 (Figure 2e and f; $p < 0.01$).

The mean aneuploidy value of all three autosomes in the Turner's syndrome samples was 14.1 ± 4.0% (range 8.9–20.6%) vs 7.2 ± 1.7% (range 4.4–9.3%) in the control samples ($p < 0.002$).

The higher mean levels of aneuploidy for each of the three autosomes in the Turner's syndrome samples consisted mostly of losses of one copy of a chromosome (monosomy) rather than gains (trisomy): 7.3 ± 3.1% vs 0.6 ± 0.5% for chromosome 8, 21.2 ± 4.9% vs 1.2 ± 2.1% for chromosome 15, and 9.8 ± 4.6 % vs 2.4 ± 1.3% for chromosome 18. When each sample was represented by the mean monosomy and trisomy values of all three autosomes, the mean values in the Turner group were 12.4 ± 3.8% and 1.4 ± 0.8%, respectively. Both values deviated significantly ($p < 0.003$ and $p < 0.02$, respectively) from the corresponding values in the control samples (6.9 ± 1.9 for monosomy, and 0.4 ± 0.3%, for trisomy) (Figure 3).

The frequency of associated autosomal aneuploidic cells in the Turner's syndrome patients was not directly related to the frequency of 45,X in the sample (compare Figure 1 with Figures 2 and 3). Specifically, samples T1–T3, with a frequency of 45,X cells above 95%, displayed a similar variation of aneuploidy for the three autosomes as that observed in all the Turner's syndrome samples, where the rate of 45,X cell frequency was much lower. Similarly, sample T4 with a frequency of more than 90% of 45,X cells showed similar or even lower levels of associated aneuploidy than samples T7 and T8 (Figure 2b, d, and f), which displayed a considerably low level (below 10%) of 45,X cells (Figure 1).

When all the Turner syndrome samples were pooled, the mean level of aneuploidy for the three autosomes in the pooled population of 46,XX cells (1804 cells) was only slightly lower ($p > 0.01$) than that found in the pooled population (3300 cells) of the 45,X cells (Figure 4). The frequency of cells with aneuploidy in the pooled population of 46,XX cells derived from the Turner patients was almost two-fold ($p < 10^{-4}$) that in the pooled population of cells (5173 cells) derived from the control subjects (Figure 4).

Discussion

Based on analyses of FISH at interphase with chromosome-specific probes for chromosomes 8, 15 and 18, the frequency of cells with aneuploidy for each of these chromosomes was significantly higher in cell samples derived from Turner's syndrome

patients than in the control subjects with normal karyotypes. Taking into account that chromosome segregation at mitosis is a multi-step process that requires proper coordination of large batteries of genes, malsegregation detected in our study suggests that in female cells, monosomic for X-chromosome, this coordination might be altered. Furthermore, our finding that a constitutional aneuploidy arising from a single event in the early life of an organism gives rise to new sporadic non-chromosome-specific losses of whole chromosomes supports the hypothesis that aneuploidy itself catalyses chromosomal instability (Duesberg & Rasnick 2000, Duesberg & Li 2003).

However, the detection of non-chromosome-specific sporadic aneuploidy in the 46,XX as well as in the 45,X cells of the Turner's syndrome patients raises the possibility that the occurrence of aneuploidy is influenced by the genetic background of the patient. Indeed, in recent mouse inbred models, it was suggested that an aneuploidy-prone background may be present in mammals, similar to what is seen in lower organisms (Bean *et al*. 2001). Evidently, an aneuploidy-prone background may evolve from various modifications in cell cycle regulators, spindle protein formation and coordination, sister chromatid separation, and anaphase segregation (Dobie *et al*. 1999, Pidoux & Allshire 2000, Shi & King 2005).

Regardless of the underlining mechanism of chromosome malsegregation in the Turner's syndrome patients, our observations are in accordance with the double aneuploidy that was previously described in Turner patients, where different mosaic cell lines including variable autosomes were detected in the same individual. In some of the cases, haplotype analyses suggested a normal zygote formation followed by several mitotic errors during early divisions, resulting in mixoploidy of 45,X/46,XX/47,XX+8 (DeBrasi *et al*. 1995). In other cases a meiotic division error of autosomal chromosome was followed by mitotic X chromosome loss, as was seen in patients with 45,X/47,XX+18 (Schubert *et al*. 2002) and 45,X/47,XX,+21 (Harada *et al*. 1998).

For all three chromosomes tested, the level of chromosome losses was much higher than the level of chromosome gains in each of the Turner's samples, suggesting that the failure is not due merely to late chromatid separation. This is in line with the results obtained in the mouse model systems prone to non-disjunction, where the frequency of chromosome losses was also significantly higher than of chromosome gains (Bean *et al*. 2001). Apparently the chance of a chromosome or chromatid getting lost in the cytoplasm from failure of a chromosome-mover component during the mitotic course is much greater than the chance of being incorporated into one of the two newly formed daughter nuclei.

The interphase FISH assay we used for detecting the copy number of a chromosome is currently the most sensitive test for detecting sporadic (low frequency <30%), non-constitutional loss and gain of a single whole chromosome (Eastmond & Pinkel 1990, Mukherjee & Thomas 1997). It is superior to metaphase spreads because of its ability to detect aneuploidy hidden in non-dividing cells, to screen large cell populations, and to avoid the loss of nucleus-free chromosomes that often occurs in the spreading of metaphase cells. Furthermore, in any given cell culture preparation, the frequency of metaphase cells is many-fold lower than that of the interphase cells.

Chromosomal instability, which makes possible continuous changes in the number of chromosomes in the diploid somatic complement, whatever the mechanism, is believed to be the cause of most malignancies (Duesberg & Rasnick 2000, Rasnick 2002, Duesberg & Li 2003). Thus, the chromosome instability observed here in Turner's syndrome genotypes is in agreement with the increased risk for cancer in these patients (Hasle *et al*. 1996, Blatt *et al*. 1997, Gravholt *et al*. 1998). Furthermore, conceptuses with aneuploidy for most autosomes, especially those carrying monosomies, are lost spontaneously in the course of pregnancy, usually due to very early developmental failure, and as such are aborted before the pregnancy is even clinically recognized (Jacobs 1990, Jacobs & Hassold 1995, Hassold & Hunt 2001). Thus, the sporadic aneuploidy is also in line with the decreased chance of a Turner's syndrome embryo crossing the pregnancy–birth barrier (reviewed in Jacobs 1990, and Jacobs & Hassold 1995).

In summary, the sporadic aneuploidy observed here in Turner's syndrome cell samples, and the large body of evidence showing that aneuploidy is an autocatalytic process (Duesberg & Rasnick 2000, Rasnick 2002, Duesberg & Li 2003), lead to the speculation that non-specific sporadic aneuploidy will accompany any genome carrying an inherent

unbalanced number of chromosomes. It may also indicate that a genetic background prone to non-proper chromosomal segregation, which gives rise to the constitutional aneuploidy, also permits the sporadic aneuploidy.

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