

Asynchronous replication of biallelically expressed loci: A new phenomenon in Turner syndrome

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Purpose: Transcriptional activity of genes is related to their replication timing; alleles showing the common biallelic mode of expression replicate synchronously, whereas those with a monoallelic mode of expression replicate asynchronously. Here the level of synchronization in replication timing of alleles was determined in subjects with Turner syndrome. **Methods:** Fluorescence in situ hybridization was used for three loci not linked to X chromosome, in lymphocytes derived from 12 controls, 3 individuals with Turner, and 4 with mosaic Turner syndrome. **Results:** In cells derived from controls, each pair of alleles replicated synchronously; yet these same alleles replicated asynchronously in cells monosomic for X chromosome derived from Turner and mosaic Turner patients. When the level of 45,X was low in the mosaic samples, the replication pattern of the 46,XX cells was normal. However, in samples with a high level of mosaicism, a significantly increased asynchronous replication was detected in the 46,XX cells. **Conclusion:** An altered temporal replication control in Turner syndrome affecting the aneuploid and euploid cells is shown. This alteration may potentially be involved in the determination of the syndrome. *Genet Med* 2002;4(6):439–443.

Key Words: Turner syndrome, mosaic, replication timing, asynchrony, fluorescence in situ hybridization

Replication patterns of known protein-encoding DNA sequences reveal a correlation between the specific time interval during the S-phase (of the cell cycle) at which a given DNA sequence in a given tissue is being replicated and its transcriptional status: expressed loci usually replicate early, while unexpressed ones replicate late. Hence, in many cell types, most housekeeping genes replicate early, whereas most tissue-specific genes demonstrate a differentiation-dependent pattern of replication, undergoing early replication in cells where they are expressed and late in cells where they are not expressed.^{1–3}

Recent fluorescence in situ hybridization (FISH) methods enable investigators to determine replication timing of allelic DNA sequences in unsynchronized cell populations.^{4,5} Accordingly, an unreplicated DNA sequence at interphase is manifested by a single fluorescent signal (singlet; S) while a replicated sequence gives rise to a double signal (doublet; D). Thus, in a population of replicating cells, a high frequency of nuclei with two similar hybridization signals, either two singlets (SS) or two doublets (DD), would indicate a pair of allelic loci that replicates synchronously. Conversely, allelic loci that replicate asynchronously are revealed by a high frequency of

nuclei containing two different hybridization signals—a singlet and doublet (SD).^{6,7}

Several studies, using the FISH method to estimate the replication timing of one allele relative to its counterpart within the same cell, have clearly shown that concomitantly expressed alleles replicate synchronously.^{6–8} In contrast, alleles subjected to an allele-specific mode of expression, due to imprinting,^{7,9–11} X chromosome inactivation,⁸ or some other mechanism leading to monoallelic expression,^{12–14} replicate asynchronously. Thus the tight association between replication timing of a gene and its transcriptional status enabled FISH methodology to be used as a reliable tool for detection of gene activity in different tissues.^{5,6,14}

New evidence based on replication studies suggests that the phenotypic abnormalities associated with an extra chromosome are caused not only by overexpression of genes present in three doses, but also by the modification of expression of other genes present in two doses. Moreover, the changes in the expression of genes present in two doses were not dependent on the source of the extra chromosome.^{15,16} In these studies it was shown that the loss of replication synchrony of three biallelically expressed genes (*RB-1*, *HER2*, and *AML1*) was more significant in amniocytes derived from subjects with trisomies 18 and 13 than from those with trisomy 21. On the other hand, cells derived from subjects carrying a sex chromosome trisomy, 47,XXX and 47,XXY, showed a normal pattern of allelic replication. Thus a correlation between the degree of replication asynchrony reflecting modification in allelic expression and the severity of the phenotypic abnormality was suggested.¹⁶

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Turner syndrome is a common disorder affecting approximately 1 per 2,500 live-born females. Among these, about 50% have a 45,X chromosomal constitution and the remainder have a second cell line containing two sex chromosomes.¹⁷ Cytogenetic studies of spontaneous abortions have shown that more than 99% of conceptions with a 45,X chromosome constitution abort spontaneously or are stillborn and only 0.3% survive to term. The reason for this is not obvious.¹⁸

In the present study we analyzed the replication pattern of allelic counterpart of *p53*, *RB-1*, and *c-myc* genes in subjects with 45,X (Turner) and mosaic 45,X/46,XX (mosaic Turner) chromosomal constitutions. The aim of this study was to determine whether genes that are not located on the X chromosome are subjected to modification in their replication pattern when present in cells carrying monosomy for the X chromosome, in comparison with cells with a normal chromosomal constitution. This study may shed light on the mechanisms leading to the Turner syndrome phenotype.

MATERIALS AND METHODS

Samples

Phytohemagglutinin (PHA)-stimulated lymphocyte cultures were evaluated from 7 patients with Turner or mosaic Turner syndrome (study group; samples 13–19) and from 12 healthy age- and sex-matched control females with normal 46,XX constitution

(samples 1–12) (Table 1). To designate the karyotype, at least 50 and 15 metaphase cells from each of the study and control samples, respectively, were examined, using G banding. The study group included three patients with 45,X karyotype, detected in all evaluated cells (samples 13–15, in those samples the presence of a Y chromosome was excluded), and four patients with mosaic Turner with variable range of X chromosome monosomy (samples 16–19) (Table 2). Two mosaic Turner patients, samples 16 and 17, revealed a low mosaicism of 45,X cells (8% and 18%, respectively), while the other two, samples 18 and 19, revealed a higher frequency of 45,X mosaicism (76% and 80%, respectively). To determine the level of 45,X mosaicism, at least 416 interphase cells were scored from each sample, using FISH with an X-chromosome-specific probe (Table 2).

Informed consent was obtained from the patients or their parents. The project was approved by the institutional review board.

Cytogenetics

Peripheral blood samples from each tested subject were incubated for short-term culture in an F10-supplemented medium in a 37°C moist chamber for 72 hours. The supplemented medium contained 20% fetal calf serum, PHA, and 1% antibiotics. After incubation, colchicine (final concentration 0.1 µg/mL) was added to the cultures for 1 hour, followed by hypotonic treatment (0.075 mol/L KCl) at 37°C for 15 minutes and

Table 1

Frequency (%) of SD cells for the designated locus in samples of normal control (46,XX) females (samples 1–12) and Turner syndrome (45,X) patients (samples 13–15)

| | Individual designation | <i>p53</i> | <i>RB-1</i> | <i>c-myc</i> | Mean ± standard deviation |
|------------------------------|------------------------|--------------|--------------|--------------|---------------------------|
| Control (46,XX) samples 1–12 | 1 | — | — | 6 | — |
| | 2 | — | — | 5 | — |
| | 3 | — | — | 7 | — |
| | 4 | — | — | 8 | — |
| | 5 | — | — | 11 | — |
| | 6 | 12 | 11 | — | — |
| | 7 | 12 | 12 | — | — |
| | 8 | 13 | 12 | — | — |
| | 9 | 10 | 11 | — | — |
| | 10 | 11 | 10 | — | — |
| | 11 | 8 | 13 | — | — |
| | 12 | 10 | 10 | — | — |
| Mean ± standard deviation | Samples 1–12 | 11 ± 2 (700) | 11 ± 1 (700) | 7 ± 2 (500) | 10 ± 2 (1900) |
| Turner (45,X) samples 13–15 | 13 | 36 (196) | 27 (189) | 31 (191) | 31 ± 5 (576) |
| | 14 | 35 (197) | 34 (197) | 33 (207) | 34 ± 1 (601) |
| | 15 | 38 (198) | 39 (197) | 38 (198) | 38 ± 1 (593) |
| Mean ± standard deviation | Samples 13–15 | 36 ± 2 (591) | 33 ± 6 (583) | 34 ± 4 (596) | 34 ± 4 (1770) |

One hundred cells were scored from each control female for each locus; the number of cells scored per locus from a Turner patient and the total number of cells scored from each group of samples are presented in parentheses.

four washes, each with a fresh cold 3:1 methanol:acetic acid solution. The lymphocyte suspensions were stored at (-4°C).

Slide preparation

Glass slides were precleaned for FISH by incubation in concentrated sulfochromic solutions, rinsed first with distilled water and then twice with absolute ethanol, and then dried with a clean cloth. The stored cell suspensions were washed with a fresh cold 3:1 methanol:acetic acid solution and then dropped onto the precleaned slides and air-dried.

Probes

Three direct-labeled commercial probes were used for the identification of the tested loci: one for the *p53* locus (17p13.1, red, Vysis 32-190008), the second for the *RB-1* locus (13q14.3, red, Vysis 32-190029), and the third for the *c-myc* locus (8q24, red, Vysis 32-190006). In addition, we used two chromosome-specific probes: one for the identification of the X chromosome (α -satellite green, Vysis 32-130029) and the second for the identification of the Y chromosome (satellite III red, Vysis 32-130025).

In situ hybridization

Fresh slide spreads were denatured for 2 minutes in 70% formamide $2 \times$ saline sodium citrate buffer (SSC) at 70°C and dehydrated in a graded ethanol series. The probe mix was then applied to air-warmed slides ($30 \mu\text{L}$ mix sealed under a 24×50 mm glass coverslip) and hybridized for 18 hours at 37°C in a moist chamber. After hybridization, the slides were washed in 50% formamide $2 \times$ SSC for 20 minutes at 43°C , rinsed in two changes of $2 \times$ SSC at 37°C for 4 minutes each, and placed in 0.05% Tween 20 (Sigma, St. Louis, MO). The slides were counterstained in 4,6-deamino-2-phenylindole (DAPI) (Sigma) antifade solution and analyzed for simultaneous viewing of fluorescein isothiocyanate, Texas red, and DAPI. An Applied Imaging system was used for the FISH analysis.

Cytogenetic evaluation

In each sample the three loci *p53*, *RB-1*, and *c-myc* were analyzed, using two-color FISH, one red for the tested locus and the second green for X chromosome identification. At least

100 interphase cells from each sample for each tested locus were recorded (Tables 1 and 2). All examined cells had two hybridization signals of the tested locus and were classified into three categories according to the replication status of the locus. Cells with two allelic counterparts at the same replication status were designated either as SS or DD, while those displaying two allelic counterparts differing in their replication status were designated SD. The SS refers to two allelic counterparts, both prior to replication, while DD indicates both counterparts after replication and SD reveals two counterparts, one prior to and the other after replication.^{4,6} The slides were blindly scored by two different and independent readers.

Statistical methods

The multiple comparisons tests (Duncan's method) were applied to test quantitative parameters between the different study groups. All tests applied were two-tailed, and a *P* value of 5% or less was considered statistically significant. The data were analyzed using the SAS software (SAS Institute, Cary, NC).

RESULTS

The replication pattern of three loci (*p53*, *RB-1*, and *c-myc*), not associated with the X-chromosome, was assessed in PHA-stimulated lymphocytes derived from 12 normal controls (samples 1–12; Table 1) and 7 individuals from the study group (samples 13–19; Tables 1 and 2).

The frequency of cells with asynchronous replication of two allelic counterparts (as indicated by the percentage of SD cells) in the control samples (46,XX) and Turner syndrome patients (45,X) differed significantly for all three loci analyzed ($P < 0.01$). The control cells had SD frequency ranging between 7 and 11 ± 2 , while the SD frequency of Turner cells ranged between 33 and 36 ± 3 (Table 1).

The 45,X cells in the four mosaic Turner patients (samples 16–19; Table 2) showed a significantly higher SD frequency in comparison with the control cells ($P < 0.01$), ranging between 29 and 42 ± 3 , and the SD frequency was not different from that of the 45,X cells of Turner samples.

The 46,XX cells in these four mosaic Turner patients showed a direct correlation between the SD frequency and the level of 45,X

Table 2

Frequency (%) of SD cells for the designated locus in monosomic (45,X) and disomic (46,XX) cell samples of mosaic Turner syndrome individuals

| Individual designation | | <i>p53</i> | | <i>RB-1</i> | | <i>c-myc</i> | | Mean \pm standard deviation | |
|------------------------|----------------------|------------|----------|-------------|----------|--------------|----------|-------------------------------|------------------|
| Sample no. | % of monosomic cells | 45,X | 46,XX | 45,X | 46,XX | 45,X | 46,XX | 45,X | 46,XX |
| 16 | 8 (1250) | 38 (100) | 8 (181) | 43 (100) | 8 (270) | 44 (100) | 9 (183) | 42 ± 3 (300) | 8 ± 1 (634) |
| 17 | 18 (555) | 41 (100) | 12 (178) | 39 (100) | 9 (174) | 43 (100) | 10 (175) | 41 ± 2 (300) | 10 ± 2 (527) |
| 18 | 76 (416) | 36 (154) | 25 (100) | 33 (147) | 18 (100) | 36 (146) | 24 (100) | 35 ± 2 (447) | 22 ± 4 (300) |
| 19 | 80 (500) | 26 (180) | 30 (100) | 32 (182) | 29 (100) | 30 (179) | 27 (100) | 29 ± 3 (541) | 29 ± 2 (300) |

Each individual is designated by its sample number (16–19) and by the percentage of the monosomic cells in its sample. In parentheses is the number of cells examined for each determination; the mean value of the three loci is presented in the last column.

mosaicism in the same sample. In samples 16 and 17, which had low levels of 45,X mosaicism (8% and 18%, respectively), the SD frequency of the 46,XX cells was 8 and 10 ± 2, and similar to the SD frequency of the control cells. However, when 45,X mosaicism levels were as high as 76% and 80% (samples 18 and 19, respectively), a significantly higher SD frequency in the 46,XX cells was detected, ranging between 22 and 29 ± 2, respectively ($P < 0.01$). Thus, in mosaic samples, not only were monosomic cells affected, but also the euploid cells residing in the same sample demonstrated a significantly increased SD frequency.

No interloci differences (for *p53*, *RB-1*, and *c-myc*) could have been demonstrated in the control, Turner, and mosaic Turner cells.

The frequency of SS (late replicating) cells in Turner (lane 4; Fig. 1) and mosaic Turner (lanes 1–3; Fig. 1) was significantly lower in comparison with the control cells (lane 5; Fig. 1), with the three loci analyzed (Fig. 1, a–c) ($P < 0.05$). Turner cells displayed SS frequency of 42–44%, the 45,X cells of mosaic Turner showed 41%, and the 46,XX cells of mosaic Turner demonstrated 45–47%. The control cells revealed a significantly higher SS frequency of 63–73%. It seems that the SD frequency was increased in Turner, 45,X, and 46,XX cells of mosaic Turner proportionally to the overall decrease in the SS frequency in these samples in the three loci analyzed.

DISCUSSION

Several mechanisms have been suggested to be involved in the determination of the stigmata of Turner syndrome. These include haploinsufficiency of escapees, i.e., genes that escape inactivation,¹⁹ level of mosaicism,¹⁸ and parent-of-origin of the X chromosome.²⁰ In this study an abnormal replication timing of autosomal genes in Turner and mosaic Turner cells was demonstrated. This phenomenon suggests an alteration that may potentially be involved in the condition.

We showed that in normally replicating cells, alleles *p53*, *RB-1*, and *c-myc* displayed a pattern of replication usually shown by concomitantly expressed alleles according to the expected Mendelian manner. In contrast, these same loci, when present in cells with a 45,X constitution in either Turner or mosaic Turner samples, displayed an early and late replication pattern, similar to alleles subjected to a process leading to monoallelic expression. Evidently, this phenomenon is not chromosome-specific, since it was observed for the *p53* locus located on chromosome 17, for *RB-1* locus mapped to chromosome 13, and *c-myc* located on chromosome 8. Therefore, chromosome X monosomy possibly modifies the replication pattern of other autosomal genes present in two doses from a biallelic mode to a monoallelic one.

This study demonstrated that euploid cells (46,XX) residing in the same sample with aneuploid cells (45,X) displayed an abnormally high SD frequency that correlated with the level of aneuploidy in that specific sample. This is suggestive of a potential interaction between the cells with a possible threshold effect dictated by the level of aneuploidy. Our findings of an increased SD frequency are in accordance with previous stud-

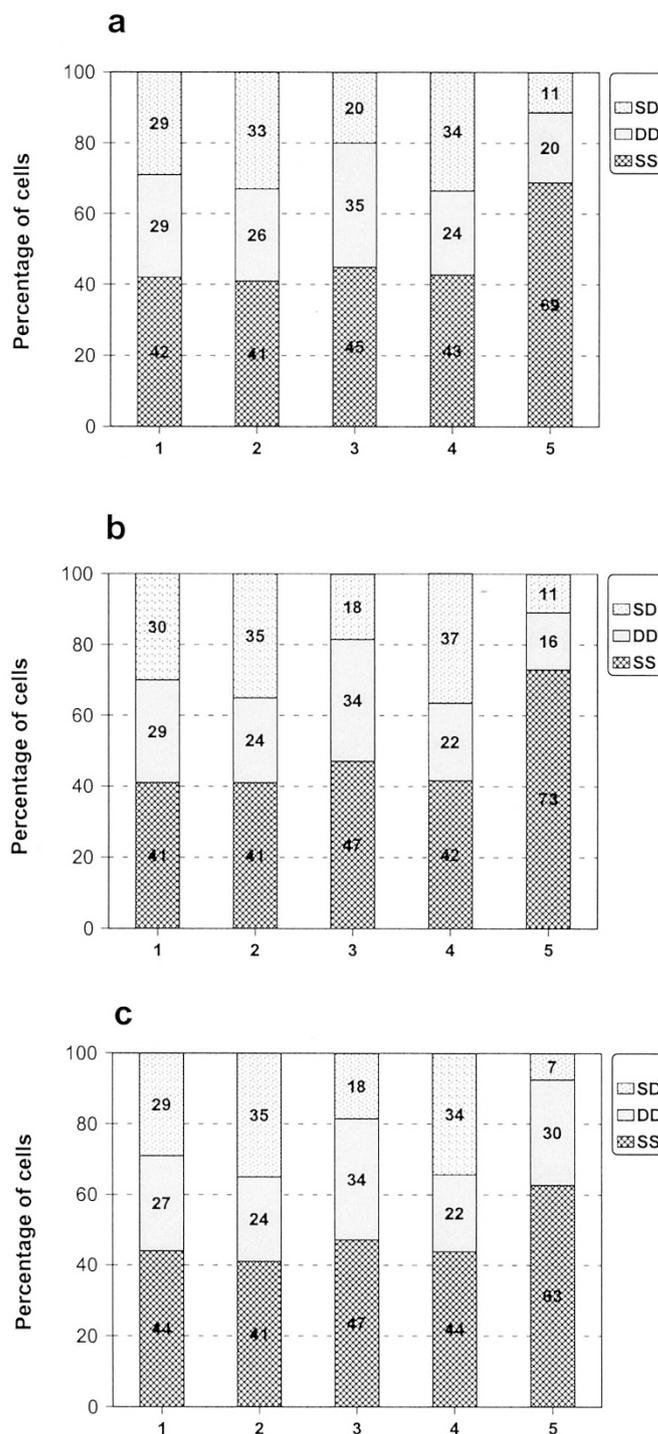


Fig. 1 Frequencies of cells containing two singlets (SS), two doublets (DD), and one singlet and one doublet (SD), following hybridization with different probes: (a) *RB-1*, (b) *p53*, (c) *c-myc*. Lanes indicate the different karyotypes: (1) mosaic Turner (45,X/46,XX); (2) 45,X cell type in mosaic Turner; (3) 46,XX cell type in mosaic Turner; (4) Turner (45,X); and (5) control group.

ies demonstrating loss of temporal replication of alleles not associated with the extra chromosome in cells derived from trisomies 21, 18, and 13.¹⁶

Although extra sex chromosome syndromes such as 47,XXX and 47,XXY did not result in asynchronized allelic mode of repli-

cation,¹⁶ in the present study it was demonstrated that X chromosome monosomy has a significantly altered replication control. Previous correlation between the degree of replication asynchrony and the severity of the phenotypic abnormality due to possible altered gene expression was suggested.¹⁶ We therefore speculate that the discrepancy in replication patterns between monosomy and extra X chromosomal constitutions may be related to the corresponding severe phenotype of Turner as opposed to Klinefelter and triple X syndromes. This suggested link between altered replication pattern, abnormal gene expression, and degree of phenotypic severity¹⁶ requires further evaluation.

Scheduled replication is essential for normal growth and development.¹⁻³ An abnormally increased asynchronous replication was detected not only in constitutional autosomal trisomies but also in several malignancies. A number of recent studies showed that the biallelically expressed genes (*p53*, *c-myc*, and *RB-1*, as well as *HER2* and *AML1*), when present in lymphocytes of individuals suffering from various types of leukemia or lymphoma, replicate asynchronously, while these same genes show a synchronous pattern of replication in lymphocytes of normal subjects.^{21,22} Moreover, such a shift from a biallelic to a monoallelic mode of replication was also reported in nonmalignant lymphocytes of renal cell carcinoma patients²³ and premalignant cells of cervical carcinoma patients.²⁴ The increased asynchronous replication of the three loci in the present study, detected in aneuploid and euploid cells derived from Turner and mosaic Turner, seems to be associated with a decrease in the SS frequency of these cells (Fig. 1). This altered SS pattern was previously shown in Down syndrome¹⁵ and chronic lymphocytic leukemia genomes,²⁵ indicating that one allele is early replicating compared to its normal time schedule. It has been previously suggested that this abnormal acceleration in replication pattern might result in activation of a normally silent oncogene.²⁵ We therefore speculate that the abnormally increased asynchronous replication demonstrated in Turner and mosaic Turner might be correlated with the altered replication pattern accompanying the cancer phenotype.^{21,22,24,26} If this altered pattern of replication proves in the future to correlate with genomic instability and abnormal gene expression, then the abnormal pattern of replication in Turner may be associated with the various malignancies detected in individuals with the condition that include colon cancer,^{27,28} neuroblastoma, and neural crest-derived tumors.²⁹

In conclusion, we demonstrated an altered replication pattern in the aneuploid cells in Turner and mosaic Turner genomes. The euploid cells showed a similar alteration when the aneuploidy level in the sample was increased. This newly detected modified replication pattern may potentially play a role in determination of the Turner condition. Our findings warrant further investigation.

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