Asynchronous Replication of Alleles in Genomes Carrying a Microdeletion

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Abstract

Background: While most allelic pairs of DNA replicate synchronously during the S phase of the cell cycle, some genes normally replicate asynchronously, i.e., genes on the X chromosome and imprinted genes. The replication control mechanism is unknown but was shown to be impaired in malignancies and chromosomal trisomies where replication pattern becomes asynchronous.

Objectives: To determine the level of asynchronization in replication timing of cells from patients with microdeleted genomes.

Methods: We applied monocolor fluorescent *in situ* hybridization with different probes on leukocytes from microdeleted genomes.

Results: All samples derived from the microdeleted genomes showed significantly higher levels of an asynchronized pattern compared to normal individuals.

Conclusions: Even a "small" genetic imbalance (microdeletion) can interfere with gene replication and cell cycle progression, as previously shown in full trisomies.

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In previous reports evaluating replication timing of alleles, we demonstrated that trisomies cause gross phenotypic disturbances. Trisomies such as 18 and 13 show the largest change in the temporal order of allelic replication, while a trisomy with a less drastic phenotypic effect, such as trisomy 21, shows a somewhat smaller change in the replication behavior of alleles. Trisomies with mild phenotypic effect such as 47,XXX and 47,XXY do not exhibit any significant change in the replication timing of alleles [1,2]. These findings suggest that the gross phenotypic abnormalities associated with an extra autosome are brought about not only by over-expression of genes present in three doses, but also by modifications in the replication of genes present in the normal two doses [3]. In the past, in order to follow the replication pattern of a given DNA sequence, cell synchronization or cell sorting was required, in addition to pre-labeling of the newly formed DNA with BrdU. Recently, a simpler and faster method was developed, based on fluorescent *in situ* hybridization, which enables determination of replication timing of allelic DNA sequences in unsynchronized cell populations [4]. Accordingly, an unreplicated DNA sequence reveals, at interphase, a single fluorescent signal (singlet, S) while a replicated sequence gives rise to a doubled signal (doublet, D). Thus, a pair of allelic loci that replicate synchronously shows a high frequency of nuclei displaying two similar hybridization signals: either two singlets (SS) or two doublets (DD). Conversely, allelic loci, which replicate asynchronously, reveal a high frequency of nuclei containing two different signals, a singlet and a doublet (SD). 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 [4–7]. The close association between replication timing and expression is best manifested by X chromosomes in cells of eutherian mammals where one X chromosome (apparently for dosage compensation) is inactivated and, as such, replicates late, while its active counterpart replicates early [8–10]. With the exception of X chromosome loci, classical replication studies showed that homologous chromosomal segments usually replicate highly synchronously [11–13]. This behavior is in accord with the simple Mendelian manner where two allelic loci are expected to be expressed concomitantly.

Using this method it was clearly shown that a pair of alleles known to be expressed concomitantly replicate synchronously [4,14,15], while alleles subjected to some mechanism leading to allele-specific expression – such as imprinting [14,16–18], methylation, X chromosome inactivation or some other allelic inactivation [2,14,19–22] – replicate asynchronously.

In the present research we examined whether there is a loss in temporal control of replication as a result of gene-dose effect associated with microdeletion contiguous gene syndromes such as Williams (7q11.23), DiGeorge (22q11.2) and velocardiofacial syndrome (22q11.2). We examined the replication pattern of the alleles RB1, p53, a locus mapped to 21q22, and c-myc in the microdeleted genomes.

Materials and Methods

Patients

We used samples of peripheral blood containing a non-synchronized population of phytohemagglutinin-stimulated lymphocytes. The samples were obtained from a patient with Williams syndrome, a patient with DiGeorge syndrome, three patients with velocardiofacial syndrome, and six healthy individuals with no history of any

Table 1. Replication pattern of control and study groups of the different loci analyzed

Probe	Group	SS	DD	SD
21q22	Control	53.5 ± 16	37.8 ± 11.5	11.1 ± 1.8
		$P > 0.05$	$P < 0.05$	$P < 0.01$
	Study	56 ± 5.8	31.0 ± 11.2	37.4 ± 4.6
Rb-1	Control	70.5 ± 10.2	29.8 ± 3.0	11.8 ± 1.0
		$P < 0.05$	$P < 0.05$	$P < 0.01$
	Study	59.4 ± 10.4	18.4 ± 6.4	26.4 ± 65.9
P53	Control	68.2 ± 16.1	38.4 ± 5.6	12 ± 1.6
		$P > 0.05$	$P < 0.01$	$P < 0.01$
	Study	63.6 ± 10.7	21.4 ± 8.8	27 ± 7.4
c-myc	Control	52.8 ± 3.8	36.7 ± 3.2	11.3 ± 1.2
		$P > 0.05$	$P < 0.05$	$P < 0.01$
	Study	45 ± 9.3	26.8 ± 2.8	24.5 ± 0.6

genetic abnormality (control group). All patients met the clinical criteria of their diagnosis, which was confirmed by molecular cytogenetic analysis (FISH). Two of the three patients with velocardiofacial syndrome were a mother and son.

Laboratory technique

Each sample was incubated in short-term culture in F10 medium supplemented with 20% fetal calf serum, 3% PHA, 0.2% heparin, and 1% antibiotics (a standard solution of penicillin and streptomycin) in a 37°C chamber. After 72 hours, colchicine (final concentration, 0.1 μg/ml) was added to the culture for 1 hr, followed by hypotonic treatment (0.075 M KCl at 37°C for 15 min) and four washes, each with a fresh, cold 3:1 methanol:acetic acid solution. The lymphocyte suspensions were stored at -20°C until use.

Slide preparation

Glass slides were pre-cleaned for FISH by incubation in a concentrated sulphochromic solution, rinsed with distilled water, followed by two series of 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 pre-cleaned slides and air-dried.

Probes

Three digoxygenine-labeled commercial probes (Oncor, France) were used, each identifying a single specific region: a) p53 for chromosome region 17p13.1 (Oncor p5106), b) RB1 for chromosome region 13q14 (Oncor, p5116), c) 21q22 for chromosome region 21q22 (Oncor, p5320), and d) c-myc for chromosome region 8q24 (Oncor p5117).

In *situ* hybridization

Fresh slide spreads were denatured for 2 min in 70% formamide 2 x SSC at 70°C and dehydrated in a graded ethanol series. The probe mix was then applied to air-warmed slides (30 ml mix sealed under a 24 x 50 mm glass cover slip) and hybridized for 18 hr at 37°C in a moist chamber. Following hybridization the slides were washed in 50% formamide/2 x SSC for 20 min at 43°C, rinsed in two changes of 2 x SSC at 37°C for 4 min each, and placed in 0.05% Tween 20 (Sigma, Israel). The slides were counterstained in DAPI (Sigma, Israel) anti-fade solution and analyzed for simultaneous viewing of FITC, Texas-red and DAPI (Chroma).

Cytogenetic evaluation

Following the application of monocolor FISH, between 54 and 140 interphase cells that showed two hybridization signals were analyzed for each given probe [Table 1]. The cells were classified into three categories according to Selig et al. [4]: a) cells with two singlets (SS), representing cells where both allelic loci are unreplicated; b) cells with two doublets (DD), representing cells in which both allelic loci have replicated; and c) cells with one singlet and one doublet (SD), revealing S-phase cells where only one of the allelic loci has replicated.

The samples were "blindly" analyzed and the frequency of cells in each category was estimated. The level of synchrony in replication timing was derived from the frequency of SD cells.

Statistical analysis

The data presented here were analyzed using the two-sample *t*-test and non-parametric test for testing differences between the study groups for quantitative parameters. The Spearman correlation was applied for testing the correlations between the study parameters examined. All tests were two-tailed, and a P value = 0.05 was considered statistically significant. The data were analyzed using the SAS software (SAS Procedures Guide, SAS/Stat User's Guide, SAS Institute Inc., North Carolina, USA).

Results

We achieved a hybridization efficiency of about 95%. The mean number of cells counted per locus was: 118 for the 21q22 locus (range 107–141), 114 for the RB-1 locus (range 98–138), 118 for the p53 locus (range 77–150), and 101 for c-myc (range 97–104).

The proportion of asynchronous cells (SD pattern) for all loci analyzed was significantly higher ($P < 0.01$) in genomes with microdeletion than in controls, with the 21q22 locus carrying the highest rate of asynchronization [Figure 1]. There was also a significant difference between microdeleted genomes and controls in the DD pattern ($P < 0.01$) for all loci analyzed that did not exist in the SS pattern [Table 1]. We found no inter-loci difference in the replication pattern among the microdeleted genomes and among the controls.

Discussion

We examined the replication pattern of six independent pairs of alleles in normal disomic cells and in viable microdeleted genomes. When present in disomic cells, each of the pairs of alleles replicated highly synchronously; thus, no difference was observed between these independent pairs of alleles. Similarly, samples from normal subjects showed no inter-individual variation in the replication pattern of alleles. It was previously shown that normal disomic cells exhibit high levels of synchrony in replication timing of homologous

FISH = fluorescent *in situ* hybridization
PHA = phytohemagglutinin

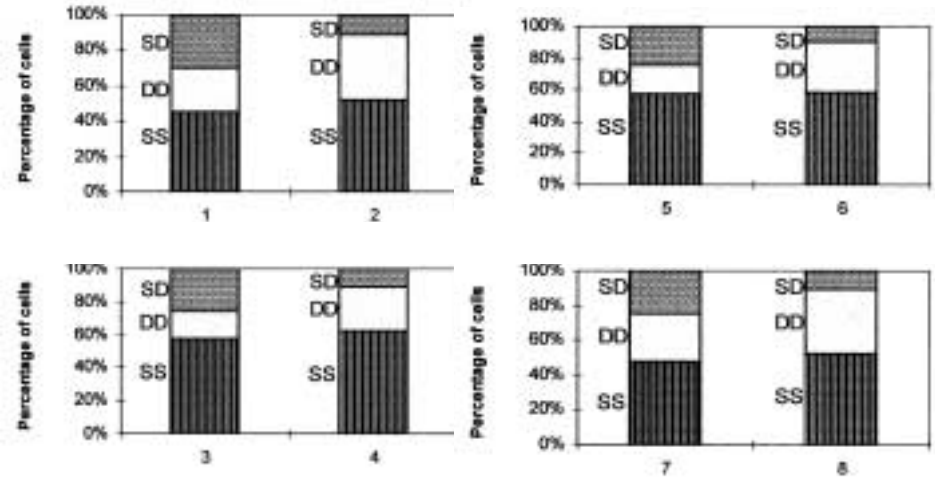

Figure 1. Replication pattern of the four different loci – 21q22, RB-1, p53 and c-myc (top to bottom). 1 = study group, uneven numbers; 2 = control group, odd numbers.

loci – both for other coding loci expressed in the expected Mendelian manner as well as for the non-coding α-satellite loci [2,20]. In contrast to normal samples, all microdeleted genomes exhibited high frequencies of asynchrony (ranging from 22 to 32). This frequency should be considered high, since the proportion of cells showing the SD pattern cannot exceed that of S-phase cells in a given cell population, which is usually around 25% in non-synchronized human cultures [23].

In normally replicating cells, the alleles tested displayed a pattern of replication usually shown by concomitantly expressed alleles according to the expected Mendelian manner. On the other hand, these same loci when present in cells carrying a microdeletion displayed an early and late replication pattern, resembling alleles subjected to a process leading to mono-allelic expression. Evidently this phenomenon is not chromosome-specific, since it was observed in p53 mapped to chromosome 17, in RB1 locus mapped to chromosome 13, in 21q22 located on chromosome 21, and in c-myc mapped to chromosome 8.

It is our understanding that synchrony in the replication timing of alleles characterizes loci expressed in the common bi-allelic mode, while asynchrony discloses loci subjected to a mechanism leading to mono-allelic expression. Therefore, our results suggest that the microdeleted status is not only associated with the lack of expression of genes normally present in the microdeleted chromosome, but it also affects the expression of other genes on other chromosomes.

Mono-allelic expression due to late or early replication of one allele is equivalent to loss of heterozygosity caused by allelic deletion – a common genetic instability associated with developmental abnormalities and malignancy [24]. Taking into account that scheduled replication is essential for normal growth and development, the loss of replication temporal control in microdeleted syndrome individual cells may play a role in the large phenotypic defects associated with the microdeleted locus in these syndromes and is not related solely to gene-dose effect. Moreover, loss of replication control was reported in cancer cells in association with loss and gain of chromosomes and in cells obtained from trisomies 13, 18 and 21 [1,25]. It may well be possible, therefore, that chromosomal unbalanced genomes interfere with the programmed differentiation-dependent replication and thus contribute to the various disorders caused by constitutional as well as sporadic aneuploidy.

Conclusion

The results of this study suggest an inconsistent affect of a microdeletion on the replication pattern of the cell. It is possible that the gene-dose imbalance caused by the microdeletion of a specific chromosome affects the replication of others, a phenomenon also seen in full trisomies and in malignancies.

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