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Replication timing of homologous α-satellite DNA in Roberts syndrome

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Abstract

Roberts syndrome (RS) is associated with a characteristic constitutive heterochromatin anomaly, namely, at metaphase the centromeres and heterochromatic segments appear split. In addition to this cytogenetic phenomenon, known as the RS effect, several other cytological features, especially affecting mitotic chromosome disjunction, are also observed. Applying FISH to interphase nuclei, we investigated the replication patterns of homologous alphoid centromeric DNA of chromosomes 9, 11, 16 and 17 in three patients showing the RS effect and in four normal individuals. A tendency for homologous centromeres to replicate asynchronously was observed in RS patients. This tendency was more evident in chromosomes 9 and 16, with large heterochromatic blocks and particularly subject to RS effect. This asynchrony could reflect a more generalized alteration in repetitive DNA replication timing that, in turn, would prevent the establishment of proper cohesion between sister chromatid heterochromatin, leading to the RS effect.

Introduction

Roberts syndrome (RS) is a rare autosomal recessive disorder characterized clinically by severe pre- and postnatal growth retardation, symmetrical limb reductions of varying severity, and craniofacial abnormalities including hypertelorism, hypoplastic nasal alae and cleft lip and palate. Approximately 80% of patients show a characteristic abnormality of constitutive heterochromatin, described as a 'puffing' or 'repulsion' of the peri–paracentromeric regions, and a splaying of the short arm of acrocentrics and of the distal heterochromatic block of the long arm of the Y chromosome (German 1979, Louie & German 1981). This cytogenetic phenomenon, known as the RS effect, is observed in cells of different tissues and appears to be more evident in chromosomes with large amounts of heterochromatin (Van Den Berg & Francke 1993).

In addition to heterochromatin 'repulsion', RS+ cells show a number of abnormal growth characteristics, including prolonged mitosis and failure to enter mitosis or proceed past metaphase (Tomkins & Sisken 1984). During anaphase, there is an increased incidence of outlying, lagging or prematurely advancing chromosomes (Louie & German 1983, Jabs *et al.* 1991). Jabs *et al.* (1991) demonstrated that lagging chromosomes account for the formation of nuclear lobulations, blebs, micronuclei and aneuploidy.

An association between DNA replication timing and rate of chromosome non-disjunction appears to exist. Litmanovitch *et al.* (1998) found that

asynchrony of centromeric DNA replication correlated with an increased risk for non-disjunction in cancer-prone individuals. The results of Amiel *et al.* (2000) pointed in the same direction, demonstrating an increased rate of allele asynchrony and aneuploidy in old women and in mothers of Down syndrome offspring. In order to investigate if centromere replication is altered in RS cells, we analysed the replication patterns of homologous alphoid DNA in interphase nuclei, through FISH.

Material and methods

Patients

Three patients with Roberts syndrome showing the typical RS effect in metaphase chromosomes (RS+ patients) were studied. According to the rating system for quantitative severity of Roberts syndrome proposed by Van Den Berghe & Francke (1993), two patients whose scores were +0.67 and +0.83, were considered severely affected, while one patient scoring +0.17 was intermediate in severity. Four normal individuals (two women and two men) were studied as controls.

FISH

FISH was performed on peripheral blood metaphases obtained after 72-h culture.

Alphoid centromeric probes pMR9a (D9Z4; Rocchi *et al.* 1991), pLC11A (D11Z1; Waye *et al.* 1987), pSE16 (D16Z2; Greig *et al.* 1989) and p17H8 (D17Z1; Waye & Willard 1986) were used.

Probes for chromosomes 9 and 16 were labelled with 14-dUTP biotin by nick translation (BioNick, BRL), while probes for chromosomes 11 and 17 were labelled with 11-dUTP digoxigenin by nick translation (Nick Translation System, BRL). FISH was performed according to Viegas-Péquignot (1992) with slight modifications. Chromosomes were denatured in 70% formamide, 2 × SSC at 70°C for 2 min and dehydrated in cold ethanol. Each probe (5–10 ng) in the hybridization mixture (50% formamide, 10% dextran sulphate, 0.1% $NaDodSO_4$–SDS, 1 × Denhardt solution in 2 × SSC) was denatured for 5 min at 100°C. Hybridization was carried out overnight at 42°C. Immunofluorescence detection was performed with FITC–avidin (1 : 25, Sigma) or antidigoxigenin rhodamine conjugated antibody (15 : 200, Boehringer Manheim) diluted in PBT (PBS, 0.1% Tween, 0.4% BSA) for 45 min at 37°C. When biotin-labelled probes were used, nuclei were stained with propidium iodide (0.4 μg/ml in PBS). Slides were mounted in Vectashield (Vector Laboratories) containing 0.2 μg/ml DAPI. Analysis was performed with a Zeiss II epifluorescence microscope. For documentation, digital images were acquired using a cooled CCD camera (PCO, VC44) and processed by means of ISIS software (MetaSystems).

The replication status of the α-satellite loci was inferred from the configuration of the hybridization signals on interphase nuclei, based on Mukherjee *et al.* (1992): (a) a compact, round or oval signal was considered as unreplicated centromeric DNA (S = single); (b) a loosely packed signal with variable shape and often grainy appearance was interpreted as decondensation of alphoid sequences during DNA replication (R = replicating), and (c) a double, rod-like or bipartite signal larger than the S signal was identified as replicated centromeric DNA (D = double).

Statistical analysis

Differences between frequencies were tested by the usual χ^2 analysis on 2 × 3 contingency tables with two degrees of freedom. For the identification of categories responsible for a significant χ^2 value, the procedure described by Haberman (1973) was applied: if the modulus (absolute value) of a given adjusted standardized residual is larger than the 5% standard normal deviation in bicaudal testing, namely 1.96, the corresponding cell is considered to be contributing significantly to the χ^2 value.

Results

Replication timing of homologous centromeres was investigated through FISH in RS+ patients and controls. Because of the prevalence of the RS effect in chromosomes with large heterochromatic blocks, chromosomes 9 and 16 were analysed, paired by size with chromosomes 11

and 17, respectively, whose heterochromatin is confined to the centromeric region. Synchrony was diagnosed when two similarly shaped signals were observed (SS, RR or DD). Two different signal configurations corresponding to successive replication stages (SR or RD) were interpreted as asynchrony with a relatively short interval between replication of homologues. Signals of non-successive replication stages (SD) indicated strong asynchrony (Figure 1). Tables 1 and 2 show the distribution of replication patterns of the centromeres of chromosome 9 and 11, and 16 and 17, respectively.

In controls, α-satellite homologous loci of chromosomes 9, 11, 16 and 17, replicated synchronously in 63.97–68.46% of metaphases. In patients, the frequencies of cells with synchronous signals on homologous centromeres were much lower (around 55%), except for chromosome 17 (63%). This decrease in frequency of cells with synchronous replication of centromeres was accompanied by an increase in frequency of cells with highly asynchronous centromere replication (SD).

In normal individuals, chromosomes 9 and 11, and 16 and 17, showed similar replication patterns of homologous centromeres (chromosomes 9 and 11, $\chi^2 = 1.063$; chromosomes 16 and 17, $\chi^2 = 0.772$). In patients, however, frequency distributions differed (chromosome 9 and 11, $\chi^2 = 12.034$; chromosomes 16 and 17, $\chi^2 = 10.186$). Asynchrony of non-successive stage type was increased and synchrony decreased in chromosomes 9 and 16 when compared with chromosomes 11 and 17, respectively (Haberman's test; chromosomes 9 and 11: $z = 3.382$ and $z = -2.057$, for asynchrony of non-successive stage type and synchrony, respectively; chromosomes 16 and

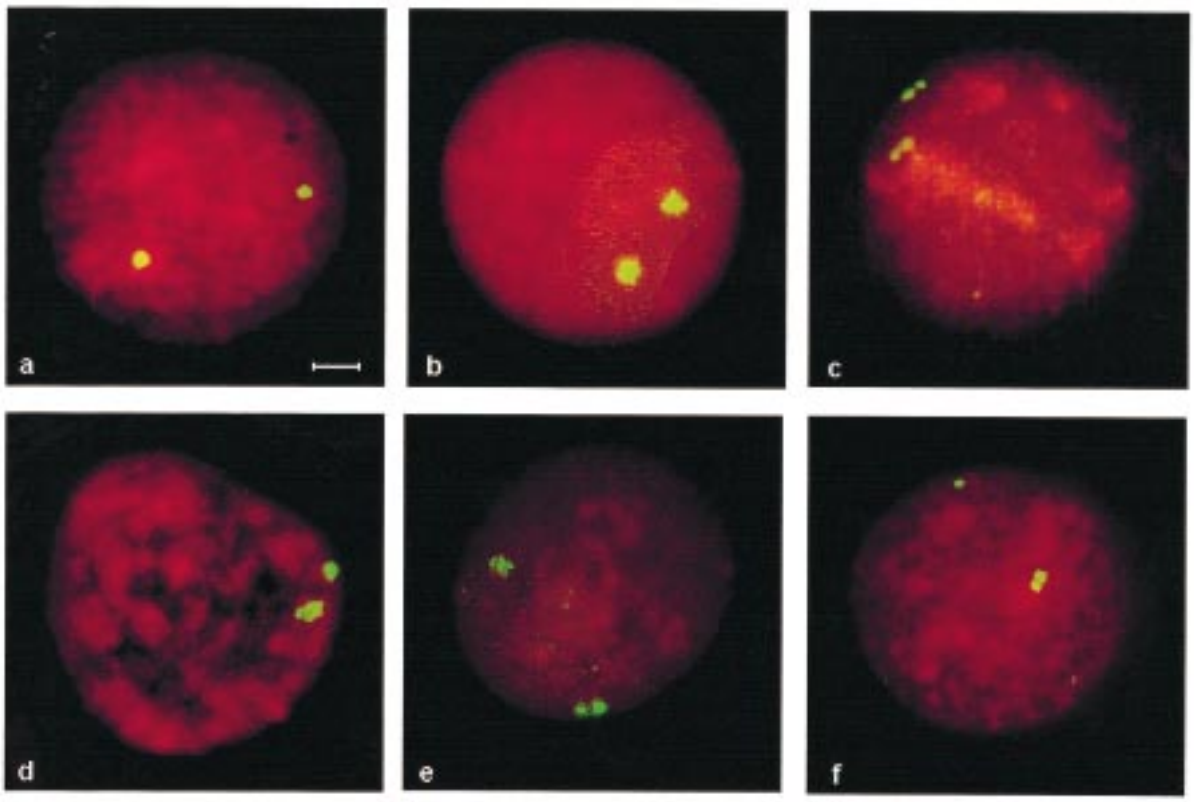

Figure 1. Fluorescent hybridization signals of alphoid centromeric probe for chromosome 16 in interphase nuclei. Two similarly shaped signals corresponding to synchronization in replication of homologous centromeres: (**a**) before replication (SS); (**b**) during replication (RR) and (**c**) after replication (DD). Signals indicating asynchrony of replication; (**d**) and (**e**) of successive stages; SR and RD, respectively; (**f**) of non-successive stages, SD. S = single; R = replicating and D = double. Scale bar = 6.3 μm.

Table 1. Replication patterns of α-satellite DNA of chromosomes 9 and 11 in interphase nuclei.

Individuals	Chromosome 9				Chromosome 11			
	Replication patterns			Total	Replication patterns			Total
	Synchrony (SS + RR + DD)	Asynchrony S[a] (SR + RD)	Asynchrony NS[b] (SD)		Synchrony (SS + RR + DD)	Asynchrony S[a] (SR + RD)	Asynchrony NS[b] (SD)	
Controls ($n = 4$)	271(65.78%)	127(30.83%)	14(3.39%)	412	280(68.46%)	119(29.10%)	10(2.44%)	409
Patients ($n = 3$)	157(52.69%)	96(32.22%)	45(15.09%)	298	186(60.98%)	99(32.46%)	20(6.56%)	305
	($z = -3.518$)*		($z = 5.575$)*		($z = -2.075$)*		($z = 2.709$)*	
	$\chi^2 = 33.522$; df = 2; $p < 0.001$				$\chi^2 = 9.176$; df = 2; $p = 0.010$			

[a]Asynchrony of successive replication stages (SR + RD); [b]Asynchrony of non-successive replication stages (SD); *Statistically significant

Table 2. Replication patterns of α-satellite DNA of chromosomes 16 and 17 in interphase nuclei.

Individuals	Chromosome 16				Chromosome 17			
	Replication patterns			Total	Replication patterns			Total
	Synchrony (SS + RR + DD)	Asynchrony S[a] (SR + RD)	Asynchrony NS[b] (SD)		Synchrony (SS + RR + DD)	Asynchrony S[a] (SR + RD)	Asynchrony NS[b] (SD)	
Controls ($n = 4$)	273(66.59%)	126(30.73%)	11(2.68%)	410	261(63.97%)	137(33.58%)	10(2.45%)	408
Patients ($n = 2$)	111(53.37%)	67(32.21%)	30(14.42%)	208	129(63.24%)	64(31.37%)	11(5.39%)	204
	($z = -3.202$)*		($z = 5.541$)*					
	$\chi^2 = 32.647$; df = 2; $p < 0.001$				$\chi^2 = 3.642$; df = 2; $p = 0.162$			

[a]Asynchrony of successive replication stages (SR + RD); [b]Asynchrony of non-successive replication stages (SD); *Statistically significant

17: $z = 3.061$ and $z = -2.031$ for asynchrony of non-successive stage type and synchrony, respectively).

Discussion

We observed a tendency for asynchrony of homologous centromere replication in RS+ patients. This was particularly prominent in chromosomes with large heterochromatin blocks.

Similar changes in replication of centromeric DNA have been described in cancer-prone individuals, coupled with an increased risk for chromosome non-disjunction (Litmanovitch *et al.* 1998). The observation that chromosome pairs whose homologous centromeres replicated highly synchronously also had low rates of aneuploidy, whereas chromosome pairs exhibiting asynchrony, with long time intervals between replicating loci, showed the highest rate of aneuploidy was taken as indicative that replication timing of α-satellite sequences is associated with centromeric function. Litmanovitch *et al.* (1998) then suggested that alphoid sequences that lose their temporal control of replication fail to fulfil their mitotic function, probably because of a failure to establish the time-dependent coordination needed for proper attachment of the CENPs. In Roberts syndrome, however, Jabs *et al.* (1991) did not detect a change in the presence or distribution of CENP-A, -B or -C. This finding associated with a normal kinetochore ultrastructure, plus the ability of RS+ chromosomes to capture microtubules led

the authors to conclude that kinetochore structural proteins were not affected in the syndrome. Indeed, a defect restricted to centromeres would not be expected as the cause of a phenomenon that affects heterochromatin even when localized far from centromeres, as is the case in Roberts syndrome.

It is possible that the asynchrony of centromere replication that we observed reflects a more generalized phenomenon affecting repetitive DNA replication as a whole. As a consequence, binding of chromatid cohesion proteins could be disturbed. Cohesins are proteins required for sister chromatid cohesion and their assembly onto chromatin is influenced by DNA replication. As pointed out by Uhlman & Nasmyth (1998), cohesins bind to specific chromosomal regions for much of interphase but they can only establish cohesion between sister chromatids during DNA replication, possibly when sister DNA molecules emerge from replication forks. In animal cells, as shown by Losada *et al.* (1998), the cohesin complex dissociates from chromosomes during prophase. However, its binding to chromosomes during interphase was shown to be essential for proper sister chromatid cohesion during mitosis, since immunodepletion of cohesins in interphase nuclei produced mitotic sister chromatid cohesion defects. Nasmyth *et al.* (2000) proposed that, in animal cells, removal of cohesins prior to sister chromatid separation could be accomplished by two separate pathways: a non-cleavage pathway would remove cohesin from chromosomes during prophase–prometaphase; a proteolytic pathway, similar to that present in yeast, would remove the remaining fraction of cohesin (or other cohesion proteins) from metaphase chromosomes, and then centromeres and other heterochromatic segments would separate. These authors suggested that centromere abnormalities in Roberts syndrome might be caused by centromeric cohesion becoming susceptible to the non-cleavage pathway. Using this line of reasoning, the asynchrony of homologous centromere replication that we observed in Roberts syndrome could reflect an alteration in repetitive DNA replication timing that would interfere with proper attachment of cohesins onto chromosomes. Cohesion of heterochromatic segments would be prematurely removed by the non-cleavage pathway and 'heterochromatin repulsion' typical of RS metaphase chromosomes would occur.

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