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Ring chromosome instability evaluation in six patients with autosomal rings

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ABSTRACT. Ring chromosomes are often associated with abnormal phenotypes due to loss of genomic material and also because of ring instability at mitosis after sister chromatid exchange events. We investigated ring chromosome instability in six patients with ring chromosomes 4, 14, 15, and 18 by examining 48- and 72-h lymphocyte cultures at the first, second and subsequent cell divisions after bromodeoxyuridine incorporation. Although most cells from all patients showed only one monocentric ring chromosome, ring chromosome loss and secondary aberrations were observed both in 48- and 72-h lymphocyte cultures and in

metaphase cells of the different cell generations. We found no clear-cut correlation between ring size and ring instability; we also did not find differences between apparently complete rings and rings with genetic material loss. The cytogenetic findings revealed secondary aberrations in all ring chromosome patients. We concluded that cells with ring chromosome instability can multiply and survive *in vivo*, and that they can influence the patient's phenotype.

Key words: Ring chromosome; Chromosome instability; Sister chromatid differentiation

INTRODUCTION

Ring chromosomes usually result from two terminal breaks in both chromosome arms, followed by fusion of the broken ends or from the union of a broken chromosome end with the opposite telomere region, leading to the loss of genetic material. Alternatively, they can be formed by subtelomeric sequence fusion or telomere-telomere fusion with no loss of genetic material, resulting in complete ring chromosomes (Henegariu et al., 1997; Sigurdardottir et al., 1999; Vermeesch et al., 2002; Le Caigne et al., 2004). Based on high-resolution molecular karyotyping, other mechanisms of the formation of ring chromosomes have been proposed, such as rings originating from an inverted duplication with a terminal deletion rearrangement (Knijnenburg et al., 2007; Rossi et al., 2008a).

In patients with ring chromosomes, further chromosomal abnormalities usually occur, due to sister chromatid exchange events during mitosis, which can result in dicentric rings, interlocked rings, and other structural conformations (Figure 1). These unstable chromosomes can lead to ring chromosome loss, producing monosomic cells, which may or may not be viable (Niss and Passarge, 1975; Ledbetter et al., 1980; Fang et al., 1995; Kosztolányi, 2009). Thus, ring chromosomes can vary in structure and number in an individual's somatic cells, resulting in a mosaic karyotype, a process called "dynamic tissue-specific mosaicism" (McDermott et al., 1977; Speevak et al., 2003).

This cytogenetic variation appears to depend mostly on ring size, rate of sister chromatid exchanges in the ring, and viability of the cell line with monosomy or with aberrant ring chromosomes (Kosztolányi, 1987a).

Ring chromosomes have been found for all human chromosomes. Usually the phenotype of ring chromosome patients overlaps that of the deletion of both ends of the respective chromosome syndromes (Schinzel, 2001). Nevertheless, the phenotypes associated with ring chromosomes are highly variable, since - in addition to the primary deletions associated with ring formation - secondary loss or gain of material may have occurred, due to the instability of ring chromosomes in general (Tümer et al., 2004; Purandare et al., 2005; Glass et al., 2006; Höckner et al., 2008; Zollino et al., 2009). Thus, the phenotype will actually depend on the size of the ring chromosome, the amount of euchromatin lost during ring formation, the ring stability, the presence of secondary aneuploid cells, and the rate of mosaicism (Kosztolányi, 1987a; Le Caigne et al., 2004).

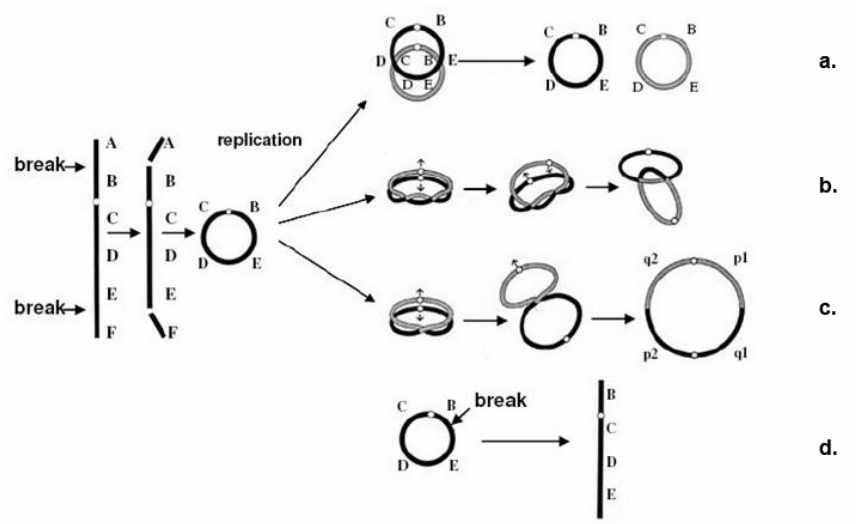

Figure 1. Scheme of ring formation and instability after replication, due to chromatid exchanges or breaks, originating: **a.** Two monocentric rings; **b.** Interlocked rings; **c.** Double-sized dicentric ring; **d.** Broken or open ring.

Cote et al. (1981) proposed the term "ring syndrome" to be used in the case of patients with apparently complete ring chromosomes who present severe intrauterine and growth retardation as the sole major physical abnormality, suggesting that the syndrome is not a consequence of the loss of genetic material but rather of cell death, due to the instability of ring chromosomes (Cote et al., 1981; Kosztolányi, 1987a). This idea was reconsidered by Rossi et al. (2008b) who believe that, at least in some ring chromosome patients, short stature is due to the haploinsufficiency of genes involved in stature. Thus, a cryptic deletion may be the basis of the phenotypic abnormalities in apparently complete rings, such as the deletion of the *IGF1R* gene at 15q26.3 in a ring 15. Also, the phenotypic variability seen in patients with similar ring chromosomes may be a consequence of their instability and of the variation in gene dosage of each cell (Hecht, 1969; Palmer et al., 1977; Zuffardi et al., 1980; Knijnenburg et al., 2007; Rossi et al., 2008a), as for instance in the patient with a ring 18 reported by Koç et al. (2008) who showed partial trisomy 18 in a considerable number of cells, due to the formation of dicentric rings, which resulted in a more severe phenotype. Rossi et al. (2008a) described a patient with a ring 13 who had a deletion and a duplication of approximately 6 Mb each, oligohydramnios and cystic kidney, features that were attributed to trisomy 13. In the present study, the instability of the ring chromosomes of six patients was investigated. The different cell lines with ring chromosomes scored were obtained from 48- and 72-h lymphocyte cultures and also from metaphase cells in the first, second or more cell divisions in culture.

PATIENTS AND METHODS

Six patients (Figure 2) with *de novo* ring chromosomes were studied.

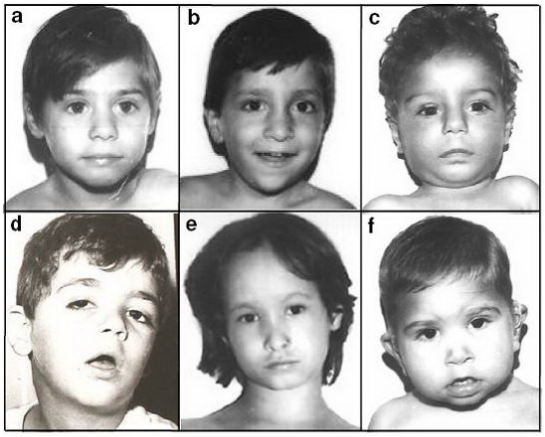

Figure 2. Frontal view of chromosomes from **a.** Patient I - ring 4; **b.** Patient II - ring 14; **c.** Patient III - ring 14; **d.** Patient IV - ring 14; **e.** Patient V - ring 15, and **f.** Patient VI - ring 18.

Patient I

Karyotype 46,XY,r(4)(p16q35). Born at term with a weight of 2200 g (<3rd centile), length of 43 cm (<3rd centile) and head circumference (HC) of 31 cm (<3rd centile). Clinical examination at 4 years and 7 months of age showed normal neuromotor and mental development, microcephaly, brachycephaly, downslanting palpebral fissures, one *café-au-lait* spot on the thorax (skin pigmentary anomaly), and bilateral retractable testis.

Patient II

Karyotype 46,XY,r(14)(p13q32). Born at term, with a weight of 2800 g (P = 10), a length of 45 cm (P = 3), and HC of 31 cm (P < 3rd centile). Clinical examination at 3 years of age revealed microcephaly, dolichocephaly, downslanting palpebral fissures, slightly prominent nose, broad nasal bridge, thin upper lip, high-arched palate, mild asymmetry of the upper central incisor teeth implantation, retrognathism, single palmar crease on the right, high anus implantation, and decreased subcutaneous tissue in the gluteus region. Skeleton X-rays revealed sacrum agenesis, hypertrophy of the muscles of the legs, club foot, prominent heels, bilateral short fourth metatarsal making the fourth toe appear short, *café-au-lait* spot in the left gluteus region, and seizures. He also had mild intellectual deficiency, limited verbal language repertoire, dysarthria, and a docile, affectionate and cooperative personality.

Patient III

46,XY,r(14)(p13q32). Born at term with a weight of 2030 g (P < 3rd centile), length 43 cm (P < 3rd centile) and HC 30 cm (P < 3rd centile). At birth, a noncyanotic congenital heart disease, severe perinatal anoxia, balanic hypospadia, sacrococcygeal pit, hypocalcemia, and hypomagnesemia were detected. At 6 months of age, interventricular communication, interatrial communication, and pulmonary stenosis with severe pulmonary

hypertension were diagnosed, in addition to microcephaly, frontal hypertrichosis, short eyelids, epicanthic folds, long eyelashes, carp-shaped mouth, thin upper lip, two longitudinal grooves on the palate, retrognathism, bilateral clinodactyly of the fifth finger, some hypochromic and *café-au-lait* spots, a pigmented nevus on the hip, right cryptorchidism, general hirsutism, seizures, intellectual deficiency, and a docile and affectionate personality.

Patient IV

46,XY,r(14)(p11q32). Born at term weighing 2900 g (10 < P < 25), measuring 46 cm (5 < P < 10) and with HC of 32 cm (3 < P < 5). Clinical examination at 9 years of age showed global muscle hypotonia, mild ataxia of trunk and limbs, brachymicrocephaly, occipital flattening, downslanting palpebral fissures, epicanthic folds, hypertelorism, esotropia, large and depressed nasal root, long philtrum, carp-shaped mouth, small and irregular teeth, high palate, large and posteriorly rotated ears, *café-au-lait* spots on the face and on the anterior-lateral side of the right forearm, and mild hirsutism. Fundoscopy showed retinitis pigmentosa. The patient displayed a hyperkinetic and puerile behavior, dysarthria and reduced verbal repertoire. He had seizures since 6 months of age.

Patient V

46,XX,r(15)(p13q26). Born at term, small for gestational age, weighing 2050 g (<3rd centile), length 45 cm (3rd centile) and HC of 30 cm (<3rd centile). Clinical examination at 8 years and 10 months of age revealed microcephaly, brachycephaly, high forehead, exotropia, hypoplastic alae nasi, high-arched palate, retrognathism, bilateral clinodactyly of the fifth finger, abnormal palmar creases, hyperextensible knees, rough and dry skin on the lower limbs, generalized hirsutism, *café-au-lait* and small hypochromic spots spread over the face and the anterior region of the chest and abdomen. An X-ray of the spine showed dorso-lumbar scoliosis. She had mild intellectual disability, reduced verbal repertoire and a docile and cooperative personality.

Patient VI

46,XY,r(18)(p11.1q23). Born at term, small for gestational age, with a weight of 2500 g (5th centile), length 45 cm (3rd centile), and HC of 33 cm (10th centile). He had recurrent gastrointestinal infections, with bouts of intermittent fever and diarrhea. Clinical examination at 9 months of age showed microcephaly, generalized hypotonia, mainly of the upper limbs, thin hair with areas of scarcity, large and posteriorly rotated ears, nystagmus, bilateral epicanthic folds, depressed nasal root, slightly anteverted nares, mouth with downturned corners, high and narrow palate, retrognathism, short neck, congenital lymphedema of the back of hands and feet, incomplete palmar crease on the right, cryptorchidism and left vertical talus.

Cytogenetic study

Lymphocytes from peripheral blood samples were obtained from 48- and 72-h mitogen-stimulated cultures, prepared according to standard cytogenetic procedures. The

slides were stained using solid staining, G- and C-banding, and also nucleolus organizer region (NOR)-staining for acrocentric ring chromosomes. For each individual, 600 metaphases were analyzed, 300 from 48-h cultures and 300 from 72-h cultures.

In other culture samples, bromodeoxyuridine (BrdU) was added at a final concentration of 10 μg/mL for the entire culture period, for sister chromatid differentiation and culture cell cycle determination. Sister chromatid differentiation staining and staging of metaphase cells as first (M1), second (M2) and third or subsequent (≥M3) mitosis in culture were performed as previously described (Melaragno and Smith, 1990). For each patient 400 cells were analyzed.

Metaphase cells were classified according to the number of chromosomes, the presence of a monocentric ring chromosome and the presence of derivative ring chromosomes (two monocentric rings, interlocked rings, double-sized dicentric ring, and broken or open ring).

For statistical analysis, the chi-square (χ^2) and the Fisher test were used, in order to compare the results from 48- and 72-h cultures, and a chi-square contingency test was applied to compare the results of M1, M2 and ≥M3 mitoses in culture.

RESULTS

G-banding revealed in patients I, II, III, and V ring chromosomes derived from chromosomes 4, 14, 14, and 15, respectively, with no apparent loss of genetic material. Patients IV and VI showed respectively a ring 14 and a ring 18, with deletion of the short arms. NOR-staining demonstrated the presence of an NOR in two of the three ring 14 patients (II and III) and in the ring 15.

Tables 1 and 2 show the total number of metaphases analyzed, as well as the number and frequency of cells with and without the ring chromosomes and of cells with secondary aberrations derived from the ring chromosome, considering the mitoses in culture.

Table 1. Number and percent of metaphase cells considering the chromosome number and the presence of secondary aberrations found in 300 cells analyzed from each of 48- and 72-h lymphocyte cultures.

Patient	Ring	Culture time	Cells with 46 chromosomes and one monocentric ring chromosome	Cells with 45 chromosomes lacking ring chromosome	Cells with secondary structural aberrations	Total cells analyzed
I	r(4)(p16q35)	48 h	286 (95.3%)	5 (1.7%)	9 (3.0%)	300
		72 h	270 (90.0%)	4 (1.3%)	26 (8.7%)	300
			$\chi^2 = 6.28$*	P = 0.7523	$\chi^2 = 8.77$*	
II	r(14)(p13q32)	48 h	293 (97.7%)	1 (0.3%)	6 (2.0%)	300
		72 h	287 (95.7%)	1 (0.3%)	12 (4.0%)	300
			$\chi^2 = 1.86$	P = 1.00	$\chi^2 = 2.06$	
III	r(14)(p13q32)	48 h	292 (97.3%)	1 (0.3%)	7 (2.3%)	300
		72 h	277 (92.3%)	16 (5.3%)	7 (2.3%)	300
			$\chi^2 = 7.65$*	P = 0.0002*	$\chi^2 = 0.00$	
IV	r(14)(p11q32)	48 h	287 (95.7%)	3 (1.0%)	10 (3.3%)	300
		72 h	274 (91.3%)	12 (4.0%)	14 (4.7%)	300
			$\chi^2 = 4.64$*	P = 0.0330*	$\chi^2 = 0.69$	
V	r(15)(p13q26)	48 h	271 (90.3%)	9 (3.0%)	20 (6.7%)	300
		72 h	286 (95.3%)	5 (1.7%)	9 (3.0%)	300
			$\chi^2 = 5.64$*	P = 1.17	$\chi^2 = 4.38$*	
VI	r(18)(p11.1q23)	48 h	277 (92.3%)	0 (0%)	23 (7.7%)	300
		72 h	274 (91.3%)	7 (2.3%)	19 (6.3%)	300
			$\chi^2 = 0.20$	P = 0.0151*	$\chi^2 = 0.41$	
Total from all patients		48 h	1706 (94.8%)	19 (1.1%)	75 (4.2%)	1800
		72 h	1668 (92.7%)	45 (2.5%)	87 (4.8%)	1800
			$\chi^2 = 6.81$*	P = 10.75*	$\chi^2 = 0.93$	

Data are reported as number with percent in parentheses.

Table 2. Number and frequency (%) of metaphase cells, considering the chromosome number and the presence of secondary aberrations, according to cell cycle in culture, found in 400 cells analyzed from each patient.

Patient	Ring	Cell cycle phase	Cells with 46 chromosomes and one monocentric ring chromosome	Cells with 45 chromosomes lacking ring chromosome	Cells with secondary structural aberrations	Total No. of cells
I	r(4)(p16q35)	M1	186 (95.9%)	1 (0.5%)	7 (3.6%)	194
		M2	99 (87.6%)	2 (1.8%)	12 (10.6%)	113
		M3/M4	81 (87.1%)	3 (3.2%)	9 (9.7%)	93
				$\chi^2 = 10.2$ (P = 0.037)		
II	r(14)(p13q32)	M1	262 (95.6%)	2 (0.7%)	10 (3.6%)	274
		M2	117 (97.5%)	0 (0.0%)	3 (2.5%)	120
		M3/M4	6 (100%)	0 (0.0%)	0 (0.0%)	6
				$\chi^2 = 1.5$ (P = 0.8255)		
III	r(14)(p13q32)	M1	122 (91.7%)	2 (1.5%)	9 (6.8%)	133
		M2	209 (91.7%)	8 (3.5%)	11 (4.8%)	228
		M3/M4	36 (92.3%)	3 (7.7%)	0 (0.0%)	39
				$\chi^2 = 6.46$ (P = 0.1673)		
IV	r(14)(p11q32)	M1	181 (96.3%)	3 (1.6%)	4 (2.1%)	188
		M2	78 (96.3%)	1 (1.2%)	2 (2.5%)	81
		M3/M4	121 (92.4%)	7 (5.3%)	3 (2.3%)	131
				$\chi^2 = 4.96$ (P = 0.2214)		
V	r(15)(p13q26)	M1	174 (95.1%)	3 (1.6%)	6 (3.3%)	183
		M2	108 (94.7%)	1 (0.9%)	5 (4.4%)	114
		M3/M4	99 (96.1%)	1 (1.0%)	3 (2.9%)	103
				$\chi^2 = 0.81$ (P = 0.9371)		
VI	r(18)(p11.1q23)	M1	155 (90.1%)	6 (3.5%)	11 (6.4%)	172
		M2	155 (95.1%)	3 (1.8%)	5 (3.1%)	163
		M3/M4	58 (89.2%)	2 (3.1%)	5 (7.7%)	65
				$\chi^2 = 3.792$ (P = 0.4352)		
Total from all patients		M1	1080 (94.4%)	17 (1.5%)	47 (4.1%)	1144
		M2	766 (93.5%)	15 (1.8%)	38 (4.6%)	819
		M3/M4	401 (91.8%)	16 (3.7%)	20 (4.6%)	437
				$\chi^2 = 8.25$ (P = 0.0828)		

Data are reported as number with percent in parentheses.

The secondary aberrations found in patients with ring chromosomes were, in decreasing order of frequency, dicentric ring chromosomes, two or three monocentric or dicentric ring chromosomes in the same cell, open rings, and chromosome fragments (Figure 3).

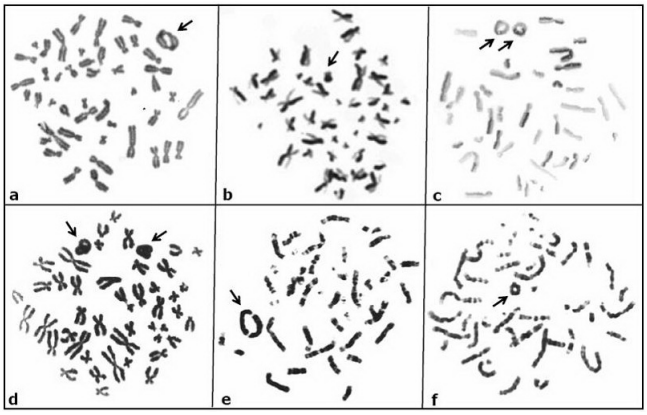

Figure 3. Metaphase cells showing ring chromosome instability: (a), (b) and (c) show cells in their first, second and third division in culture using BrdU incorporation. Arrows show ring chromosomes varying in number and morphology: (a, e) dicentric ring 4, (b) opened ring 14, (c) two dicentric rings 14, (d) two monocentric rings 4, (f) dicentric ring 18.

DISCUSSION

In all patients, most cells analyzed (90 to 97.7%) showed only one monocentric ring chromosome, as previously described in different reports concerning 48- and 72-h cultures of patients with ring chromosomes. The percentage of cells with 45 chromosomes due to ring loss and of cells with secondary structural aberrations varied from zero to 5.3 and from 2.0 to 8.7, respectively.

In a study by Kosztolányi (1987a), a ring chromosome was considered to be "stable" when secondary aberrations were found in 0-5% of the mitoses and "unstable" when such aberrations occurred in more than 5% of the mitoses counted.

We found that the cells showing ring chromosome instability can vary among different ring chromosomes (4, 14, 15, and 18) and even when the ring chromosome is the same, as shown by our three ring 14 cases, a finding also reported by Chitayat et al. (1987).

In 48-h cultures, the ring 4 and all ring 14 chromosomes were considered to be stable, whereas rings 15 and 18 were unstable. In 72-h cultures, ring 4, two of the three ring 14 chromosomes and ring 18 can be considered unstable. We found no clear correlation between ring size and ring instability, as suggested by Lejeune (1967) and Kosztolányi (1987a), and no difference between apparently complete rings and rings with genetic material deletion.

In patients I, II and IV, we found a smaller number of cells with one monocentric ring in the 72-h cultures compared to the 48-h cultures, along with an increase in the frequency of cells showing instability of the ring chromosomes; yet, in patient V, we observed the opposite. When we scored the cells after BrdU incorporation for cell cycle differentiation, we found a significant alteration in the different cell types only regarding patient I (ring 4), who showed a decrease in the percentage of cells with one monocentric ring chromosome with each cell generation in culture.

Tsukino et al. (1980) and Riley et al. (1981) also observed an increase in the frequency of monosomic cells for the ring chromosomes as the lymphocyte culture time increased. Tsukino et al. (1980) attributed this finding to ring chromosome instability *in vivo*, while Riley et al. (1981) considered them as resulting from *in vitro* ring loss, since they believed that these cells were not viable *in vivo*.

Cote et al. (1981), using BrdU for cell cycle determination in 48- and 72-h cultures, found secondary aberrations exclusively in cells after two cell cycles in culture, and suggested that these cells would not survive *in vivo* and that the frequency of cells showing secondary aberrations in the second cell cycle in culture would be the same as the rate of cell death *in vivo*.

Kosztolányi and Pap (1986) and Kosztolányi (1987b) studied two cases of ring chromosomes (ring 4 and ring 15) that were shown to be unstable both in lymphocyte and fibroblast cultures. An increased cell death rate was detected by cell viability determination with Trypan blue exclusion in fibroblast cultures. Since ring derivative chromosomes could also be seen in lymphocytes after only one cycle in culture, the authors suggested that such cells are also generated *in vivo* and that behavioral instability of rings at mitosis probably occurs *in vivo* as well.

A variation in the stability of ring chromosomes was also found among different tissues. Some researchers found higher frequencies of monosomic cells without the ring chromosomes in fibroblast compared to lymphocyte cultures (Sparkes et al., 1967; Moore et al., 1973; Palmer et al., 1977; Peeden et al., 1983), while others found no significant differences between the two tissues (Valente et al., 1977; Ledbetter et al., 1980; Manouvrier-Hanu et al., 1988). Ledbetter et al. (1980), however, found ring chromosomes 15 with an abnormal morphology in 9% of metaphase cells from lymphocyte cultures and in 20 and 24% of cells from fibroblast cultures in the third and tenth subculture, respectively, although it was not possible to know if these cells were formed *de novo* or were perpetuated in a clonal manner. Manouvrier-Hanu

et al. (1988) also found a higher frequency of cells with two ring chromosomes 9 in fibroblast compared to lymphocyte cultures.

Cote et al. (1981) and Kosztolányi (1985) suggested that cells with secondary aberrations would not survive *in vivo*, where they would be eliminated in the following cell divisions. Different conclusions were drawn by Hernandez et al. (1979), who found similar frequencies of cells containing only one ring 13, of monosomic cells and of cells with different types of ring chromosomes in 48-, 72- and 96-h lymphocyte cultures, and also by de Almeida et al. (1983) who found no monocentric ring chromosome 13 in either 48- or 72-h lymphocyte cultures and who concluded that these chromosomes were unlikely to be produced *in vitro*.

In the present study, cytogenetic data revealed in all ring chromosome patients the presence of secondary aberrations in 48- and 72-h lymphocyte cultures and also in metaphase cells that were in different cultured cell generations. Considering both our data and those from the literature, we believe that cells derived from ring chromosome instability can multiply and survive *in vivo*, and may have an influence on the phenotypic variations of patients with ring chromosomes.

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