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TITLE

Altered bivalent positioning in metaphase I human spermatocytes from Robertsonian translocation carriers

ABSTRACT

Purpose: To determine whether there is an altered bivalent positioning in metaphase I human spermatocytes from Robertsonian translocation carriers.

Methods: Metaphase I human spermatocytes from three 45,XY,der(13;14)(q10;q10) individuals and a 45,XY,der(14;15)(q10;q10) individual were analyzed. Proximity relationships of bivalents were established by analyzing meiotic preparations combining Leishman staining and Multiplex-FISH procedures. Poisson regression model was used to determine proximity frequencies between bivalents and to assess associations with chromosome size, gene density, acrocentric morphology and chromosomes with heterochromatic blocks. The hierarchical cluster Ward method was used to characterize the groups of bivalents with preferred proximities in a cluster analysis. Bivalent groups obtained were individually compared with those obtained in normal karyotype individuals evaluated in a previous study.

Results: A total of 1,288 bivalents were examined, giving a total of 2,289 proximity data. Only four positive significant proximities were detected for each type of Robertsonian translocation. Significant bivalent associations were only observed by small-size chromosomes for MI,22,XY,III(13q14q). These results were clearly divergent from 46,XY individuals. Moreover, cluster analysis revealed that about 30% of the bivalents showed changes in their proximity relationships in metaphase I.

Conclusions: The territorial organization of bivalents in metaphase I human spermatocytes changes in the presence of a Robertsonian translocation.

KEYWORDS

Chromosome territories, metaphase I, meiosis, Robertsonian translocation, spermatocytes

1 INTRODUCTION

2 Chromosomes occupy specific regions of the nuclear space called chromosome territories (CTs)
3 [1]. Nuclear spatial arrangement of CTs has been related to some chromosomal parameters
4 such as gene density [2–5] or chromosome size [6]. Moreover, the distribution pattern of CTs is
5 distinct, depending on the cell type [7,8].

6 The distribution of CTs has functional significance in terms of maintenance and regulation of
7 the genome [9]. It has been suggested that specific nuclear architecture may regulate gene
8 expression [10–12]. Chromosome positioning could promote chromosomal interactions
9 between specific regions of the genome to activate or repress their expression [10,11]. Actually,
10 alterations in the spatial arrangement of chromosomes have been associated with various
11 diseases. Cremer et al. (2003) described different patterns of positioning for chromosomes 18
12 and 19 between normal and tumor cell lines [13]. A change in the position of chromosome 17
13 territory has also been described for cells infected with Epstein-Barr virus [14]. Meaburn et al.
14 (2007) described changes in territories for chromosomes 13 and 18 in patients with *LMNA*
15 mutations [15].

16 Little is known about the maintenance and distribution of chromosomes during the cell cycle.
17 Although some authors have proposed that territorial chromosome organization is maintained
18 throughout the cell cycle [16], others suggested that this organization is re-established in the
19 early G1 phase [17,18].

20 Meiotic studies of chromosome positioning have demonstrated a non-random chromosome
21 distribution in spermatogenesis. Some authors have revealed a preferential proximity of the
22 bivalent 15 to the XY pair in human spermatocytes during the pachytene stage [19] and a
23 preferential proximity location of bivalents 15 and 22 to the sex bivalent at metaphase I (MI)
24 [20]. Studies of bivalent positioning at MI have demonstrated a non-random distribution of
25 chromosomes and have observed preferred associations depending on chromosome size,
26 chromosome morphology and gene density [21].

27 Several publications have reported a non-random spatial organization of chromosomes in the
28 nuclei of spermatozoa [22–26]. Sperm chromosome position could be crucial in the
29 decondensation and remodeling of chromatin domains and consequently, achieve an
30 epigenetic control of gene expression in the embryo [26–28].

31 In humans, Robertsonian translocations are one of the most common structural
32 reorganizations. They are detected in 1/1000 newborns [29], but this percentage is nine times
33 higher in infertile patients [30]. The reduced fertility in Robertsonian translocation carriers is
34 mainly due to the formation of chromosomally abnormal sperm as a result of the regular

segregation of the chromosomes involved in the reorganization and interchromosomal effects phenomena (ICE). Moreover, Robertsonian translocations would be associated with a reduction in gamete production resulting from the activation of meiotic checkpoints triggering apoptosis [31]. Some studies have shown that the presence of chromosomal rearrangements alters the positioning of chromosomes in the sperm nucleus [32–34]. This observation has been related to changes in the expression profile of paternal alleles in the embryo [26–28]. Altogether, this suggests that alterations of sperm chromosomal territoriality could be an additional cause of infertility. In this work, we have determined the effect of a Robertsonian translocation on the territorial organization of bivalents in MI. To achieve this aim, we compared the relative position of all bivalents in MI in a group of Robertsonian translocation carriers to individuals with normal karyotypes.

MATERIALS AND METHODS

Biological Samples

Semen samples (P1, P2) or testicular biopsies (P3, P4) were obtained from four Robertsonian translocation carriers: three individuals 45,XY,der(13;14)(q10;q10) (P1, P2, P3) and one 45,XY,der(14;15)(q10;q10) (P4). Patients gave their informed consent with regard to the participation in the study and protocols were approved by our Institutional Ethics Committee. Seminogram analyses, according to the World Health Organization (WHO) criteria [35], showed: oligoasthenoteratozoospermia (P1, P3), oligoasthenozoospermia (P2) and teratozoospermia (P4). Data from testicular tissue samples used as controls have been detailed in a previous study [21].

Semen samples P1 and P2 exhibited high numbers of spermatogenic cells at different meiotic stages, which is quite common in samples from infertile males [36]. Accordingly, these samples were used for downstream analyses. Additionally, testicular tissue samples were obtained for patients P3 and P4.

In any case, samples were incubated in a hypotonic solution (KCl 0.075 M) at 37°C. Biopsies were mechanically disaggregated to obtain a cell suspension. Semen samples and meiotic cell suspensions were centrifuged, and the pellets were fixed in methanol:acetic acid (3:1). Fixed material was dropped onto dry slides and kept at –20°C until chromosome analysis.

Bivalent Identification

Leishman stain diluted at 20% in a buffer solution prepared according to Weise was used to stain the chromosome preparations (Fig. 1a). The evaluation was carried out with an Olympus

BX60 microscope (Olympus Optical España S.A.) equipped with a capture and image analysis system (CytoVision 2.7, Applied Imaging). MI images were captured and coordinates were noted. Leishman-stained slides were destained before being processed for M-FISH (Spectra Vysion Assay, Vysis Inc.) according to a protocol optimized in our laboratory (Sarrate et al. 2004). As a result, bivalents were labeled with a combination of five different fluorochromes, obtaining 24 different color patterns, one for each bivalent (Figs. 1b and c). Thus, M-FISH allowed a karyotype to be created (Fig. 1c), facilitating metaphase proximity analysis by comparing the images captured after the M-FISH protocol with those previously obtained after Leishman staining.

Proximity Analysis

All metaphases included in this study were MI,22,XY,III(13q14q) or MI,22,XY,III(14q15q), exhibiting all bivalents, including the XY pair and the chromosomes involved in the reorganization as a trivalent. In each cell, nearby bivalents were determined for each bivalent, including chromosomes involved in the reorganization which were each individually analyzed. We considered those that form the first ring around a specific bivalent to be “nearby”.

Proximity was established for the 253 bivalent pair possibilities ($C[23,2] = \frac{1}{2} \times 23 \times 22 = 253$ combinations of two without repetitions). Nearby bivalents were scored as “1” and the absence of proximity as “0”. As a consequence, bivalents involved in the reorganization were always scored as “1”. From these values, tables of proximity for each metaphase were built (Appendix Table 1). The addition of data from all of the analyzed metaphases allowed the creation of proximity tables for MI,22,XY,III(13q14q) (Appendix Table 2) and MI,22,XY,III(14q15q) (Appendix Table 3).

Statistical Analysis

In order to determine proximity frequencies between bivalents, a generalized regression model with repeated measures considering a Poisson distribution [37] was performed separately for 45,XY,der(13;14)(q10;q10) and 45,XY,der(14;15)(q10;q10) individuals. Additional regression models were used to assess whether chromosome size, morphology, gene density or the presence of heterochromatic blocks determined bivalent positions. Wherever possible, the groups of chromosomes analyzed were set from the five most representative chromosomes of each parameter evaluated (Table 1). Chromosomes involved in the rearrangement were excluded from the acrocentric group to prevent the bias caused by the constant proximity of these chromosomes in a trivalent configuration. A second analysis was performed with the aim of grouping nearby chromosomes. This was done by a multidimensional scaling (MDS) analysis [38] that summarizes proximities between

chromosomes by a set of quantitative variables used in a hierarchical cluster analysis. Results were represented in a tree dendrogram using R-squared distance. Clustering results were compared to those defined in individuals with normal karyotypes and published elsewhere [21]. Briefly, bivalents were distributed in three clusters in normal karyotypes: Cluster I constituted larger size chromosomes (1;2;3;4;5;6;7;8;9;10;11;12); Cluster II was mainly defined by the presence of acrocentric chromosomes (13;14;15;21;22;XY;18); and Cluster III was characterized by rich-gene chromosomes (16;17;19;20). Differences were calculated by taking into account the percentage of bivalents remaining in the same cluster and those that changed. The concordance between classifications was calculated by Cohen's Kappa coefficient.

RESULTS

A total of 1,288 bivalents were examined, giving a total of 2,289 proximity data. Only four positive significant proximities were detected for each type of Robertsonian translocation (Table 2). Contrasting these results with proximities described in normal karyotype MI analysis [21], only two of the four bivalent pairs per type of Robertsonian translocation were coincident (Table 2). There were 20 bivalent pairs with significant proximities in normal karyotype MI analyses [21] that did not remain statistically significant for both translocations studied. Proximity analysis of specific bivalent groups revealed statistically significant nearby locations of small-size chromosomes for MI,22,XY,III(13q14q). Neither of the other bivalent groups showed significant proximities in MI,22,XY,III(13q14q), nor any bivalent group with specific features in MI,22,XY,III(14q15q) (Table 1). Cluster analysis defined three bivalent groups of preferred proximity in MI,22,XY,III(13q14q) (Fig. 2a) and MI,22,XY,III(14q15q) (Fig. 2b). Comparative analysis between clusters obtained in this study and clusters obtained from normal karyotype individuals showed a difference of 35% for MI,22,XY,III(13q14q) and 30% for MI,22,XY,III(14q15q) with a Kappa value of 0.47 (CI = 0.16 to 0.73) and 0.55 (CI = 0.31 to 0.79), respectively.

DISCUSSION

Our study provides evidence that the territorial organization of bivalents in MI changes in the presence of a Robertsonian translocation. We observed an alteration of bivalent pairs with significant proximities compared to those described in normal karyotype individuals, and an alteration of the relationship between chromosome features and bivalent positioning. There are different non-mutually exclusive possibilities to explain such variations. Studies in Robertsonian translocation carriers have described that the reorganized chromosomes usually

show asynaptic regions at pachytene which are often associated with asynaptic regions of other bivalents [39–44]. It has also been described that asynaptic regions suffer heterochromatinization and gene silencing [45,31]. Moreover, Circularized Chromosome Conformation Capture (4C) studies have shown that active and inactive chromatin are found in different nuclear domains [46]. Therefore, heterosynaptic regions at prophase I could alter chromosome positions according to the chromatin state of activation and this altered position could be maintained at least until the MI stage. Another consequence of heterosynapsis is the alteration of the frequency and distribution of chiasmata in the rearranged chromosomes [47–50], as in other chromosomes [50–53]. In agreement with this, MI included in this study showed a lower mean chiasmata count than MI in the normal karyotype population [54]. This reduction was of six chiasmata in the MI of Robertsonian translocation carriers for 13,14 and of four chiasmata in Robertsonian translocation carriers for 14,15 (excluding chiasmata of reorganized bivalents). Although the number and distribution of chiasmata is crucial to bivalent orientation in the meiotic spindle, it has been demonstrated that preferential proximities between bivalents are not affected by chiasmata count variations [21].

The second possibility could be related to the characteristics of acrocentric chromosomes. They have short arms composed of tandem copies of ribosomal DNA (rDNA) genes called nucleolar organizing regions (NORs). These regions are specifically associated during interphase to form the nucleolus [55]. Since the genesis of a Robertsonian translocation requires the loss of the short arms, derivative chromosomes may not associate successfully to form the nucleolus. Thus, the relative position between acrocentric chromosomes could be disturbed in interphase and the same positioning alteration could be still present in MI.

To conclude, our results provide the first evidence of a chromosome territoriality alteration in the presence of a Robertsonian translocation in MI human spermatocytes. Considering the accumulating evidence supporting a functional significance of chromosome territoriality, the characterization of CTs during spermatogenesis could open new insights to determine whether there is a relationship between chromosome positioning, genome regulation and male infertility.

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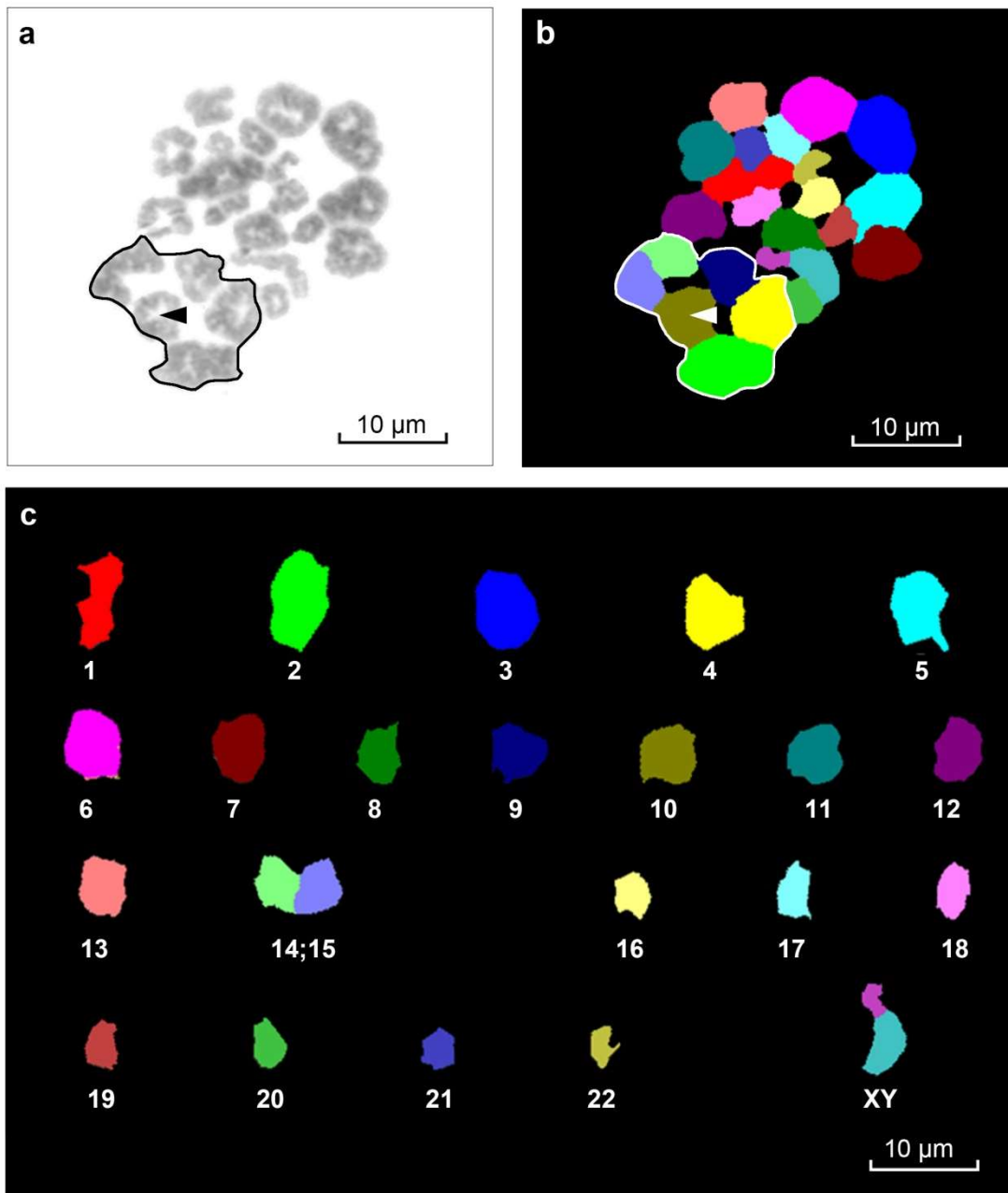
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1 **FIGURES**

2



3

4

5 **Fig. 1** MI in Leishman staining (a) and M-FISH (b) with the corresponding M-FISH karyotype
 6 MI,22,XY,III(14q15q) (c). Identification of the nearby bivalents that constitute the first ring around the
 7 bivalent 10 (indicated with an *arrowhead*): 2;4;9;14;15 (a,b).

8

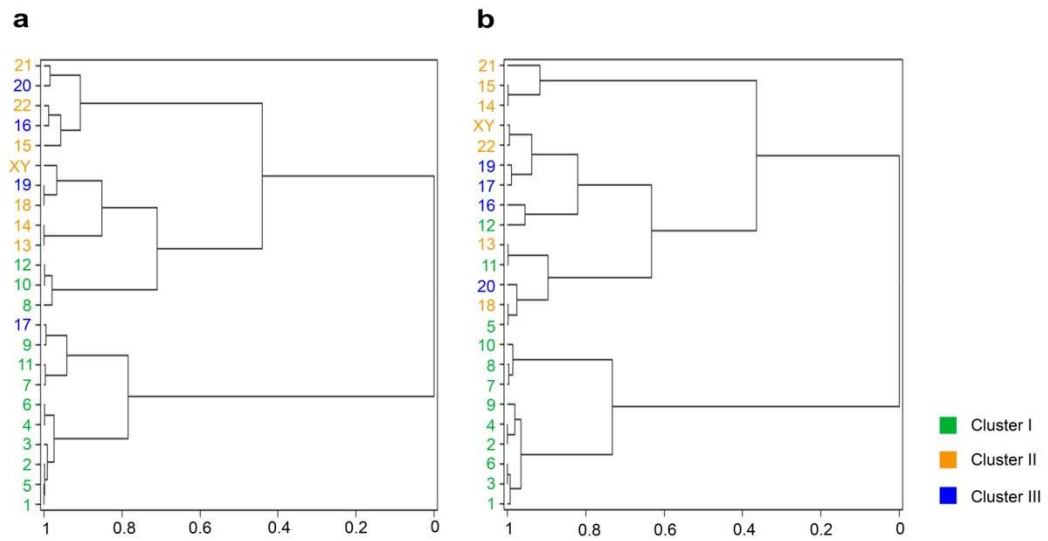


Fig. 2 Dendrogram representation of bivalent proximities for MI,22,XY,III(13q14q) (a) and MI,22,XY,III(14q15q) (b). Different colors indicate groups of chromosomes resulting from clustering of MI control data [21]

Note: MI,22,XY,III(13q14q) Kappa value = 0.47; CI = [0.16–0.73]

MI,22,XY,III(14q15q) Kappa value = 0.55; CI = [0.31–0.79]

1 **TABLES**
2

			Confidence Limit		P-value	Proximity ^a
Chromosome feature	Bivalents	%	Lower	Upper		
MI,22,XY,III(13q14q)	Large-size chromosomes	(1;2;3;4;5)	12.9	10.1	16.5	0.452
	Small-size chromosomes	(18;19;20;21;22)	19.3	17.3	21.5	0.0233
	High-density chromosomes	(11;16;17;19;22)	15.1	13.0	17.6	0.950
	Low-density chromosomes	(4;5;8;13;18)	16.1	14.8	17.6	0.318
	Acrocentric chromosomes	(15;21;22)	15.2	14.8	15.6	0.688
	Chromosomes with heterochromatic blocks	(1;9;16)	17.9	14.7	21.8	0.487
MI,22,XY,III(14q15q)	Large-size chromosomes	(1;2;3;4;5)	12.0	7.6	19.0	0.080
	Small-size chromosomes	(18;19;20;21;22)	12.7	8.1	19.8	0.127
	High-density chromosomes	(11;16;17;19;22)	16.7	11.3	24.7	0.799
	Low-density chromosomes	(4;5;8;13;18)	19.3	13.4	27.8	0.593
	Acrocentric chromosomes	(13;21;22)	17.8	11.9	26.5	0.940
	Chromosomes with heterochromatic blocks	(1;9;16)	24.4	13.6	44.1	0.291

3
4 **Table 1** Analysis of associations among preferential bivalent group proximity and chromosome features
5 of MI,22,XY,III(13q14q) and MI,22,XY,III(14q15q)
6 *Note:* MI,22,XY,III(13q14q) global mean = 15.2; CI = [14.7–15.7]
7 MI,22,XY,III(14q15q) global mean = 17.5; CI = [16.2–18.9]
8 ^a Preferential bivalent proximities (+)
9
10
11
12
13

		Confidence Limit		
	Bivalents	Mean (%)	Lower (%)	Upper (%)
MI,22,XY,III(13q14q)	*Chr20;21	39	24	64
	*Chr15;22	34	20	58
	Chr20;XY	34	20	58
	Chr16;20	29	17	52
MI,22,XY,III(14q15q)	*Crh11;18	47	22	98
	*Crh22;XY	47	22	98
	Crh3;XY	47	22	98
	Crh5;18	47	22	98

14
15 **Table 2** Significant bivalent pairs associations of MI,22,XY,III(13q14q) (n = 41) and MI,22,XY,III(14q15q)
16 (n = 15) *Note:* Global mean = 0.152; 95% CI = [0.147–0.157]
17 *Significant bivalent pairs coinciding with those described in normal karyotypes [21]
18

APPENDIX
2

Cr.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	XY
1		0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	1	1	0	0	1	1	0
2			0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
3				0	1	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0
4					0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	1	0	0	1
5						0	1	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0
6							0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	1	0
7								0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1
8									1	0	0	0	0	0	0	1	0	1	1	0	0	0	1
9										1	0	1	0	1	0	0	0	1	0	0	0	0	1
10											0	0	0	1	1	0	0	0	0	0	0	0	0
11												1	1	0	0	0	0	0	0	0	1	0	0
12													0	1	0	0	0	1	0	0	0	0	0
13														0	0	0	1	0	0	0	1	0	0
14															1	0	0	0	0	0	0	0	0
15																0	0	0	0	0	0	0	0
16																	0	1	1	0	0	1	0
17																		0	0	0	1	1	0
18																			0	0	0	1	0
19																				0	0	0	1
20																					0	0	1
21																						0	0
22																							0
XY																							

Table 1 Count example proximities between the bivalents in MI,22,XY,III(14q15q)

- 4
- 5
- 6
- 7
- 8
- 9
- 10
- 11
- 12
- 13
- 14
- 15
- 16

Cr.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	XY
1		4	2	6	8	5	9	9	8	8	7	3	6	6	8	6	7	7	6	6	4	4	7
2			6	1	7	10	4	6	3	4	5	6	7	8	2	5	10	5	6	5	7	3	6
3				11	2	6	5	4	7	4	5	7	6	5	9	9	3	9	4	4	10	4	7
4					6	9	3	7	9	1	7	7	6	2	6	4	7	7	8	0	5	3	9
5						2	9	4	11	8	11	4	6	6	3	7	5	7	7	7	5	9	3
6							7	6	2	3	11	4	3	6	5	5	6	10	5	7	5	4	8
7								7	8	7	7	3	2	3	5	7	11	5	6	3	10	5	7
8									5	11	3	3	8	5	4	8	4	9	3	5	11	7	5
9										10	9	6	4	4	8	8	6	4	8	9	5	4	9
10											3	9	6	7	6	7	5	7	8	11	7	4	10
11												4	6	1	7	7	11	6	2	6	3	4	5
12													5	7	9	5	5	10	6	7	9	9	8
13														41	3	7	5	6	3	3	2	6	10
14															4	4	3	4	8	5	1	8	6
15																8	8	9	8	9	8	14	11
16																	2	6	6	12	7	10	7
17																		3	6	5	10	7	6
18																			9	4	4	10	5
19																				4	8	7	8
20																					16	9	14
21																						8	6
22																							6
XY																							

Table 2 Incidence counts of proximities between the bivalents in MI,22,XY,III(13q14q) (n = 41)

2

3

4

5

6

7

8

9

10

11

12

13

14

15

Cr.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	XY
1		1	1	3	1	5	4	3	4	2	5	3	0	2	1	3	6	2	5	2	3	2	2
2			2	4	0	3	5	3	4	5	4	1	2	4	2	3	1	0	1	2	3	0	3
3				3	2	5	3	1	3	2	4	1	0	3	3	3	2	1	1	2	3	4	7
4					1	2	5	2	5	2	2	2	3	1	2	2	3	3	2	2	5	1	3
5						1	4	2	1	2	4	6	2	1	1	5	3	7	2	4	2	2	5
6							2	3	2	1	1	3	3	6	3	3	3	2	2	0	6	5	2
7								5	7	6	2	1	2	0	3	2	4	1	4	2	3	3	4
8									2	4	2	3	4	2	1	1	2	1	5	2	2	1	4
9										6	1	1	1	2	3	4	5	2	2	0	0	4	3
10											0	2	4	4	2	2	3	3	2	3	0	1	1
11												2	5	2	1	3	2	7	1	5	2	2	2
12													4	1	2	4	1	2	1	3	2	2	5
13														1	1	2	4	4	2	3	3	3	4
14															15	3	0	2	2	1	3	2	3
15																3	1	1	3	1	6	2	3
16																	0	4	3	2	1	4	4
17																		5	4	1	6	4	3
18																			2	3	2	3	4
19																				1	1	2	2
20																					1	1	4
21																						3	1
22																							7
XY																							

Table 3 Incidence counts of proximities between the bivalents in MI,22,XY,III(14q15q) (n=15)

2

3

4

5

6