Journal of Medical Genetics, 1983, **20**, 350–356

C heterochromatin variation in couples with recurrent early abortions

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SUMMARY The possible influence of the high polymorphic C heterochromatic regions of human chromosomes 1, 9, 16, and Y on meiotic chromosome segregation was investigated. Faulty chromosome segregation may be the result of either an abnormal quantity of C heterochromatin on the homologues, or disequilibrium between the homologues. The aim of our study was to determine whether either a variation in the amounts of total C heterochromatin or differences in the amounts of C heterochromatin between homologues could lead to faulty chromosome segregation. The study was performed on C banded metaphases obtained from peripheral lymphocyte cultures of 15 couples with recurrent early abortions and 15 control couples, all Caucasians.

Analysis of variance was first performed on separate metaphases to measure intra-individual, inter-individual, and between population variation in a hierarchical model. Since the significant intra-individual differences covered the other parameters we performed, secondly, a one way analysis of variance on the mean values of metaphases per person in order to measure the inter-individual and between population variation. The results did not show a relationship between C heterochromatin lengths and occurrence of recurrent abortions.

Polymorphism of C heterochromatic regions is a well established fact in plants and animals, including the C heterochromatic regions of chromosomes 1, 9, 16 (secondary constriction), and Y (distal part of the long arm) in man.[1–6]

Although this variation seems to have no phenotypic influence, it is quite possible that there exists an influence on the mechanism of cell division.[1 2] By inhibiting the formation of chiasmata, C heterochromatin variants may cause inefficient formation of bivalents which lies at the origin of unequal chromosome segregation.[7–9] This involves not only chromosomes 1, 9, and 16 but also the chromosomes with which they are associated, namely the acrocentrics, because associated chromosomes would segregate in an associated way.[10–13] It is known that early miscarriages are mainly the result of chromosome abnormalities in the fetus as a consequence of faulty chromosome segregation in the gametes at the first meiotic division.[14–17] Therefore, we studied the variability of C heterochromatic regions in couples with recurrent abortions at an early stage of fetal growth. Numerous studies have been done on this subject but the results are controversial. In some a relationship between C heterochromatin variation and recurrent abortions, sterility, lowered fertility, etc, has been found,[7 18–23] and in some it has not.[24–27] Because of this controversy we reconsidered the problem in a very detailed way with objective length measurements of the C heterochromatic regions, using a large number of cells from each person, and making a powerful statistical analysis of the results.

Received for publication 7 July 1982.
Accepted for publication 18 March 1983.

Materials and methods

PATIENT SELECTION

All persons examined were Caucasians. The control population consisted of 15 couples with two or more healthy children and no known miscarriages. The affected population consisted of 15 couples with two or more early miscarriages referred to a genetics laboratory because there was no indication of any gynaecological defect.

CULTURE METHOD AND STAINING

Lymphocyte cultures were made according to the (slightly modified) method of Moorhead *et al.*[28] Phytohaemagglutinin and colcemid were used for stimulation of the lymphocytes and metaphase arrest, respectively. Five days after harvest the metaphases were C banded according to Sumner.[29] During the preparation, the chromosomes may be exposed to influences which could modify their morphological properties. To avoid these effects as much as possible, the techniques for collecting, treating, and staining the preparations have been strictly standardised.

METHODS OF MEASUREMENT

C banded metaphases were photographed and good quality metaphases (about 20 or 25 per person) were selected. Only well spread metaphases with visually the same degree of contraction and with chromosomes 1, 9, 16, and Y in a straight position were used. The length of chromosomes 1, 9, 16, and Y (in the male population) was measured using a Hewlett Packard (X, Y) digitiser and co-ordinates of four points for each chromosome were recorded. These were the start and end of the chromosome (P1 and P4) and the start and end of the C band (P2 and P3).

For the Y chromosome the end of the C heterochromatic region and of the whole chromosome is the same (P3 = P4). These coordinates were transmitted to a CDC computer system which calculates length values from the coordinates. The results were expressed as relative C heterochromatin values, namely the length of C heterochromatin as a function of the length of the total chromosome for each chromosome of each metaphase. The accuracy of this method was recently demonstrated in our laboratory by Staessen *et al.*[30] It takes into account the degree of differential condensation of the metaphases in the same preparation, the degree of differential condensation of each chromosome in the same metaphase, and the difference in contraction between euchromatin and C heterochromatin, as reported by Robson *et al.*[31]

STATISTICAL METHOD

The original data were transformed either into C heterochromatin values averaged over both homologues $\left(\frac{H+H'}{2}\text{ value}\right)$ for each chromosome pair in each metaphase, or into mean C heterochromatin values for each chromosome of each person (M value).

Statistical analysis was performed by analysis of variance. Firstly a hierarchical model was used which measures intra-individual (between metaphases of the same person), inter-individual (between persons of the same population), and between population (between the different populations, namely control couples and couples with recurrent abortions) variation of the $\frac{H+H'}{2}$ values for each chromosome pair in each metaphase. Secondly, a one way model of variance was used which measures the intra-individual and between population variation of the M values for each chromosome of each person.

Results of the Kolmogorov-Smirnoff tests (normality of distributions) and Barlett tests (homogeneity of variances) revealed that the basic criteria for performing analysis of variance were met.

Results

Table 1 gives the results of the analysis of variance (hierarchical model) performed on the relative C heterochromatin values averaged over both homologues $\frac{(H+H')}{2}$ separately for the chromosome pairs 1, 9, and 16. Analysis was performed separately for males and females. The values obtained for each

TABLE 1 *Analysis of variance (hierarchical model) of relative C heterochromatin values (homologues averaged) for chromosomes 1, 9, and 16 in control parents (CP) and couples with recurrent abortions (RA).*

	Females		*Males*	
	df	*Mean squares*	*df*	*Mean squares*
Chromosome 1*				
Within persons, between metaphases	679	0·113‡	668	0·127‡
Between persons of the same status†	28	0·067	28	0·069
Between status CP/RA	1	0·001	1	0·022
Residual	709	0·001	698	0·001
Chromosome pair 9*				
Within persons, between metaphases	679	0·211‡	668	0·224
Between persons of the same status	28	0·084	28	0·118
Between status CP/RA	1	0·040	1	0·049
Residual	709	0·002	698	0·002
Chromosome pair 16*				
Within persons, between metaphases	679	0·253‡	668	0·268‡
Between persons of the same status	28	0·079	28	0·101
Between status CP/RA	1	0·045	1	0·071
Residual	709	0·002	698	0·002

*The chromosome pair is analysed by calculating for each metaphase the average over both homologues $\frac{(\text{homologue} + \text{homologue}')}{2}$.

†Status refers to control parents (CP) or couples with recurrent abortions (RA).

‡Indicates highly significant variation ($p < 0·001$).

TABLE 2a *Mean relative C heterochromatin lengths in the males of the control parents and the couples with recurrent abortions, with M and M′ indicating the mean values for both homologues separately.*

Sample	*Case*	*No of metaphases*	*M1*	*M1′*	*M9*	*M9′*	*M16*	*M16′*	*MY*
Control population	C1	23	0·25±0·02	0·22±0·02	0·34±0·04	0·28±0·04	0·39±0·04	0·34±0·04	0·62±0·07
	C2	25	0·25±0·05	0·22±0·05	0·34±0·07	0·30±0·06	0·38±0·06	0·34±0·06	0·61±0·07
	C3	19	0·24±0·04	0·22±0·04	0·35±0·05	0·30±0·05	0·38±0·05	0·32±0·05	0·50±0·06
	C4	15	0·31±0·04	0·27±0·04	0·37±0·06	0·34±0·05	0·41±0·05	0·34±0·05	0·67±0·06
	C5	21	0·27±0·04	0·23±0·09	0·34±0·05	0·29±0·04	0·38±0·04	0·31±0·04	0·61±0·07
	C6	20	0·25±0·04	0·22±0·04	0·35±0·05	0·25±0·05	0·38±0·05	0·34±0·05	0·58±0·08
	C7	50	0·26±0·03	0·21±0·04	0·36±0·05	0·32±0·05	0·36±0·06	0·31±0·04	0·60±0·06
	C8	15	0·24±0·02	0·21±0·03	0·30±0·03	0·27±0·04	0·36±0·04	0·33±0·04	0·56±0·05
	C9	19	0·23±0·04	0·19±0·05	0·31±0·04	0·27±0·03	0·34±0·04	0·30±0·04	0·60±0·06
	C10	24	0·26±0·03	0·20±0·04	0·42±0·04	0·34±0·04	0·39±0·04	0·34±0·06	0·62±0·06
	C11	20	0·20±0·03	0·18±0·03	0·29±0·04	0·23±0·04	0·30±0·04	0·26±0·04	0·53±0·06
	C12	20	0·22±0·03	0·18±0·03	0·29±0·05	0·24±0·03	0·33±0·04	0·27±0·06	0·53±0·08
	C13	25	0·17±0·02	0·14±0·02	0·25±0·03	0·22±0·03	0·26±0·03	0·22±0·04	0·50±0·05
	C14	18	0·21±0·04	0·18±0·02	0·32±0·05	0·27±0·05	0·36±0·04	0·31±0·04	0·56±0·05
	C15	21	0·20±0·02	0·18±0·03	0·32±0·05	0·27±0·04	0·38±0·03	0·33±0·03	0·57±0·07
	Mean population value		0·24±0·03	0·20±0·03	0·33±0·04	0·28±0·03	0·36±0·04	0·31±0·03	0·58±0·05
Affected population	A1	16	0·20±0·02	0·18±0·02	0·30±0·04	0·22±0·06	0·32±0·05	0·28±0·04	0·55±0·06
	A2	35	0·26±0·04	0·22±0·03	0·37±0·05	0·31±0·04	0·37±0·04	0·32±0·04	0·59±0·06
	A3	27	0·25±0·03	0·23±0·03	0·38±0·05	0·32±0·05	0·39±0·04	0·34±0·03	0·65±0·06
	A4	19	0·25±0·03	0·20±0·04	0·33±0·04	0·29±0·04	0·38±0·05	0·32±0·05	0·65±0·07
	A5	26	0·26±0·04	0·21±0·03	0·30±0·04	0·25±0·04	0·32±0·03	0·28±0·03	0·53±0·07
	A6	13	0·29±0·04	0·24±0·02	0·40±0·03	0·34±0·05	0·41±0·04	0·38±0·04	0·63±0·06
	A7	26	0·26±0·04	0·21±0·04	0·34±0·04	0·29±0·04	0·37±0·03	0·31±0·05	0·62±0·06
	A8	18	0·20±0·03	0·17±0·02	0·29±0·05	0·25±0·04	0·30±0·03	0·27±0·05	0·47±0·05
	A9	30	0·22±0·03	0·19±0·03	0·30±0·04	0·27±0·03	0·34±0·03	0·29±0·04	0·54±0·07
	A10	14	0·18±0·02	0·15±0·02	0·25±0·03	0·21±0·03	0·28±0·03	0·25±0·03	0·48±0·06
	A11	28	0·21±0·04	0·19±0·03	0·29±0·03	0·26±0·04	0·33±0·04	0·29±0·04	0·57±0·05
	A12	33	0·22±0·02	0·19±0·02	0·34±0·03	0·29±0·03	0·33±0·09	0·29±0·04	0·59±0·05
	A13	29	0·22±0·04	0·19±0·03	0·31±0·04	0·28±0·04	0·36±0·05	0·30±0·05	0·59±0·06
	A14	26	0·19±0·03	0·17±0·03	0·29±0·04	0·26±0·04	0·33±0·05	0·28±0·06	0·56±0·06
	A15	25	0·23±0·03	0·21±0·02	0·35±0·04	0·30±0·03	0·38±0·05	0·32±0·05	0·58±0·06
	Mean population value		0·23±0·03	0·20±0·02	0·32±0·04	0·28±0·03	0·35±0·04	0·30±0·03	0·57±0·05

TABLE 2b *Mean relative C heterochromatin lengths in the females of the control parents and the couples with recurrent abortions, with M and M′ indicating the mean values for both homologues separately.*

Sample	*Case*	*No of metaphases*	*M1*	*M1′*	*M9*	*M9′*	*M16*	*M16′*
Control population	C1	25	0·25±0·03	0·23±0·03	0·38±0·04	0·31±0·03	0·38±0·04	0·35±0·05
	C2	23	0·24±0·04	0·27±0·03	0·33±0·05	0·29±0·06	0·37±0·04	0·31±0·05
	C3	27	0·25±0·04	0·22±0·04	0·34±0·04	0·29±0·05	0·38±0·06	0·33±0·04
	C4	26	0·25±0·04	0·22±0·04	0·34±0·05	0·29±0·04	0·38±0·07	0·32±0·05
	C5	39	0·24±0·04	0·20±0·04	0·34±0·04	0·30±0·05	0·37±0·06	0·32±0·05
	C6	20	0·26±0·04	0·20±0·04	0·33±0·05	0·28±0·04	0·36±0·05	0·33±0·05
	C7	15	0·26±0·04	0·23±0·03	0·33±0·03	0·28±0·04	0·39±0·04	0·32±0·04
	C8	17	0·20±0·02	0·17±0·02	0·30±0·05	0·25±0·04	0·34±0·05	0·28±0·04
	C9	16	0·25±0·03	0·23±0·02	0·33±0·05	0·25±0·03	0·38±0·03	0·32±0·03
	C10	25	0·20±0·03	0·18±0·03	0·29±0·05	0·24±0·04	0·34±0·05	0·29±0·04
	C11	25	0·19±0·03	0·16±0·03	0·30±0·04	0·25±0·03	0·31±0·05	0·28±0·05
	C12	20	0·22±0·03	0·19±0·03	0·30±0·04	0·26±0·05	0·34±0·03	0·27±0·04
	C13	25	0·19±0·03	0·16±0·02	0·27±0·04	0·24±0·03	0·27±0·04	0·23±0·04
	C14	20	0·23±0·03	0·20±0·03	0·30±0·05	0·26±0·04	0·35±0·04	0·30±0·04
	C15	16	0·22±0·03	0·20±0·03	0·28±0·04	0·24±0·04	0·35±0·05	0·30±0·04
	Mean population value		0·23±0·03	0·20±0·02	0·32±0·03	0·27±0·02	0·35±0·03	0·30±0·03
Affected population	A1	25	0·28±0·03	0·19±0·04	0·30±0·03	0·26±0·04	0·32±0·05	0·29±0·05
	A2	22	0·29±0·04	0·25±0·03	0·39±0·05	0·34±0·04	0·41±0·04	0·35±0·04
	A3	30	0·26±0·03	0·22±0·03	0·40±0·05	0·32±0·04	0·38±0·05	0·32±0·04
	A4	29	0·24±0·05	0·20±0·04	0·34±0·05	0·29±0·05	0·37±0·06	0·31±0·05
	A5	19	0·24±0·05	0·21±0·04	0·33±0·05	0·29±0·03	0·37±0·04	0·30±0·05
	A6	25	0·22±0·03	0·19±0·02	0·33±0·05	0·28±0·05	0·38±0·05	0·33±0·04
	A7	15	0·24±0·05	0·20±0·04	0·36±0·05	0·28±0·05	0·34±0·04	0·29±0·05
	A8	35	0·18±0·03	0·16±0·02	0·29±0·03	0·25±0·03	0·29±0·04	0·25±0·03
	A9	24	0·21±0·03	0·18±0·02	0·30±0·04	0·25±0·03	0·35±0·04	0·30±0·04
	A10	17	0·17±0·03	0·14±0·02	0·29±0·04	0·25±0·04	0·32±0·04	0·27±0·03
	A11	31	0·24±0·04	0·21±0·03	0·33±0·05	0·29±0·04	0·35±0·05	0·29±0·04
	A12	31	0·25±0·03	0·19±0·04	0·32±0·04	0·27±0·04	0·32±0·04	0·27±0·05
	A13	27	0·23±0·04	0·21±0·05	0·30±0·04	0·27±0·04	0·34±0·05	0·30±0·05
	A14	20	0·22±0·02	0·20±0·02	0·28±0·04	0·22±0·04	0·33±0·04	0·28±0·04
	A15	21	0·25±0·04	0·21±0·03	0·30±0·06	0·31±0·05	0·34±0·05	0·31±0·05
	Mean population value		0·23±0·03	0·20±0·02	0·33±0·03	0·28±0·03	0·35±0·03	0·30±0·02

TABLE 3 *Analysis of variance (one way) of mean relative C heterochromatin values per person for both homologues considered separately, for the homologues averaged* $\frac{(M+M')}{2}$, *or for the difference between homologues* $(M-M')$.

	df	*Mean squares*							
		Homologues separately				*Homologues averaged*		*Difference between homologues*	
		M female	*M male*	*M′ female*	*M′ male*	$\frac{(M+M')}{2}$ *female*	$\frac{(M+M')}{2}$ *male*	$(M-M')$ *female*	$(M-M')$ *male*
Chromosome 1*									
Within persons of the same status†	28	0·009	0·0011	0·0006	0·0007	0·0007	0·0009	0·0002	0·0001
Between status CP/RA	1	0·0001	0·0004	0·0000	0·0003	0·0000	0·0004	0·0002	0·0000
Chromosome 9*									
Within persons of the same status	28	0·0011	0·0016	0·0007	0·0013	0·0009	0·0014	0·0001	0·0002
Between status CP/RA	1	0·0008	0·0005	0·0007	0·0004	0·0007	0·0004	0·0000	0·0000
Chromosome 16*									
Within persons of the same status	28	0·0009	0·0014	0·0007	0·0011	0·0008	0·0012	0·0001	0·0001
Between status CP/RA	1	0·0004	0·0011	0·0003	0·0003	0·0004	0·0010	0·0000	0·0000
Chromosome Y*									
Within persons of the same status	28						0·0026		
Between status CP/RA	1						0·0003		

*The chromosomes are analysed by calculating for each person the relative C heterochromatin value of the homologues separately, of the average over both homologues, and of the difference between both homologues.
†Status refers to control parents (CP) or couples with recurrent abortions (RA).
The absence of ‡ indicates that no significant differences were found.

cell measured are taken into account in this kind of analysis. Values obtained for mean squares show that no statistically significant inter-individual or between population variation was obtained. However the intra-individual variations are highly significant for each chromosome pair in both males and females. These results indicate that the average relative C heterochromatin over both homologues of a chromosome pair is highly variable in different metaphases of the same person.

Table 2a and b shows the mean C heterochromatin values of each chromosome in each person (for males and females respectively). M and M′ are used to distinguish between the two homologues of a chromosome pair. The homologue with the highest relative C heterochromatin value is referred to as M and the homologue with the lowest value as M′. When the difference between the two homologues could not be visualised, the classification into larger and smaller was based on the results from the measurements.

Table 3 gives the results of the analysis of variance (one way variance model) performed on the M and M′ mean relative C heterochromatin values per person. The analysis of variance was performed on the homologues separately, on the homologues averaged, and on the difference between the two homologues. The values obtained for the different mean squares are all below the level of significance. The results given in this table are those of the analysis performed on males and females separately. When males and females were considered together (as couples) similar results were obtained.

Discussion

The relationship between the occurrence of recurrent abortions and the presence of C heterochromatin variants has been analysed in different studies. A survey of those studies is given in table 4. These studies vary in the number of couples examined, in the number of metaphases analysed per person, in the method for the estimation of C heterochromatin values (from subjective scoring to objective measuring methods), and finally in the power of the statistical methods applied. The results obtained are therefore controversial.

Our purpose was to reconsider the problem with special attention to the number of metaphases scored per person (about 20 to 25), the accuracy of the measuring method (a length measuring method was applied and the C heterochromatin is expressed as a relative C heterochromatin value[30]), and the power of the statistical method (analyses of variance were performed to provide a quantitative evaluation of the intra-individual, inter-individual, and between population variation of the C heterochromatin).

The variability of the mean relative C heterochromatin value separately for each chromosome pair (1, 9, and 16) was analysed first by a hierarchical model of analysis of variance (table 1). This analysis compared the intra-individual variation, the inter-individual variation within a population (control parents vs couples with recurrent abortions), and the between population variation of the mean relative C heterochromatin value separately for each homologue (1, 9, and 16) averaged in each metaphase. It is clear from this analysis that for both males and females and for the three chromosome pairs studied, only the intra-individual variation is statistically highly significant. The inter-individual and the between population variation was negligible in comparison. This means that even when a large

TABLE 4 *Survey of studies estimating the relationship between the variation of C heterochromatin and the occurrence of recurrent abortions, infertility, etc.*

Reference	*No of persons*	*No of cells per person*	*Measuring method*	*Statistical method*	*Relationship between C heterochromatin variation and recurrent abortions, infertility, etc*
Holbek *et al*[18]	80 couples with recurrent abortions	5	Subjective	χ^2	+
Nielsen *et al*[7]	8712 persons	?	Subjective	Fisher	+
Patil and Lubs[21]	4400 newborn infants	5–10	Subjective	χ^2	+
Ford[22]	185 male partners of childless couples, 45 fertile	4	Subjective	χ^2 and Spearman rank test	+
Mayer *et al*[24]	1050 persons	?	Subjective	χ^2	−
Hemming and Burns[25]	50 couples with recurrent abortions 50 control parents	2	Objective	χ^2	−
Beltran *et al*[26]	18 fathers of abnormal sons 27 fathers of normal sons	5	Objective	Correlation and regression	−
Brown *et al*[27]	231 persons 39 couples with a normal baby 38 couples with an abnormal baby	5	Objective	Analysis of variance (hierarchical model)	−
Ford *et al*[23]	58 female partners with recurrent abortions 100 control women	1	Objective	Mann-Whitney two tailed test Analysis of variance Z test	+

number of metaphases is taken into account, the difference between the C heterochromatin lengths of the chromosomes in the different metaphases of the same person is of such importance that it covers the other variation parameters.

This high intra-individual variation is the result of the differential degree of contraction between euchromatin and heterochromatin, between the chromosomes of one metaphase and between the metaphases of one preparation. This biological phenomenon is difficult to avoid, so this kind of analysis cannot make any conclusions about the importance of between population variation. As we examined a large number of metaphases per person with an accurate measuring method, the mean C heterochromatin value of those metaphases would provide as good as possible an estimation for the C heterochromatin value of each chromosome in each person.

Therefore, instead of considering each metaphase separately, the data were analysed in a second step (one way analysis of variance) starting from a mean C heterochromatic value per person. The value used is an estimator for each chromosome of each person and is of course dependent on the number of metaphases examined, in our study about 20 to 25 per person. Most other authors also use mean values per person, but the number of metaphases studied in the different samples was variable and usually rather small (from one to ten metaphases per person) (see table 4). We suggest that the use of a C heterochromatin estimator based on a small number of metaphases could be, at least partially, an explanation for the controversy about the existence or absence of a relationship between C heterochromatin variants and the occurrence of recurrent abortions. Our analysis, based on a relatively large number of metaphases per person, failed to demonstrate any relationship between C heterochromatin variants and recurrent abortions. Neither the quantity of C heterochromatin (on each homologue separately or averaged over both homologues) nor the difference between the two homologues (expressed as the difference between the two homologues) differ significantly between the control and the affected populations, suggesting that the non-disjunction phenomena are not caused by any of these factors. One cannot exclude that small differences may exist between the two samples, but we were not able to demonstrate them. However, if important differences had been present, the method described here would probably have shown them.

We thank the 'Laboratoires de Cytogénétiques' of Dr Vamos, the 'Centre Génétique' of Drs Hayez and Van Regenmorter, and Dr Van der Stappen for kindly providing the blood samples. We also thank the people in our own laboratory for all the help they gave us during this study.

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