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The Role of Cytologic NOR Variants in the Etiology of Trisomy 21

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Summary

Silver-stained chromosomes from 29 couples with a trisomy 21 offspring and from 25 control couples were studied to determine whether there was an association of nucleolar-organizing-region variants in parents of children with trisomy 21. A reproducible scoring system for the analysis of silver-stained chromosomes was developed, and this was applied to the analysis of study participants in a blinded fashion. Seven of the 58 parents of children with trisomy 21 and seven of the 50 control parents were found to have variant NORs on silver staining. Therefore, we do not find a demonstrable risk for nondisjunction of chromosome 21 in individuals with silver-staining variants.

Introduction

Couples who have had one trisomy 21 child have an increased risk for additional affected children, suggesting there may be genetic factors that contribute to the etiology of nondisjunction (Carter and Evans 1961; Uchida 1970; Richards 1977; Alfi et al. 1980). Discovery of such factors would be of great importance, as this may permit identification of couples at increased risk for having an affected child.

Numerous attempts to identify genetic factors predisposing to trisomy 21 have been reported in the literature. A number of these studies have focused on abnormalities of the short arm of the acrocentric chromosomes (Dekaban et al. 1963; Hamerton et al. 1965; Edgren et al. 1966; Starkman and Shaw 1967; Sands et al. 1969). More recently, silver-staining variants of the nucleolar organizing region (NOR) were reported to be more frequent among parents of children with trisomy 21 (Jackson-Cook et al. 1985). That study suggested that these cytologic variants could be used to identify individuals at increased risk for meiotic nondisjunction.

In this paper, we describe a reproducible scoring system for the analysis of silver-stained chromosomes, and we present the results of a blinded, case-control cytogenetic study to determine the frequency of the NOR variants in parents of children with trisomy 21 and in control couples.

Material and Methods

Selection of Study Participants and Specimen Collection

Twenty-nine families with a trisomy 21 child were identified from records at the Clinical Genetics Center of The Children's Hospital of Philadelphia. The 25 control couples consisted of individuals receiving medical services at The Children's Hospital of Philadelphia or the Hospital of the University of Pennsylvania. Family histories of all control individuals were carefully screened, and potential control individuals were excluded from the study if there was a history of trisomy 21 in a close relative, if there was a history of multiple spontaneous abortions, or if family history was otherwise suggestive of a chromosome abnormality. Each

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control couple had at least one healthy child. The 25 control couples had a total of 59 living children, 10 spontaneous abortions, and one each ectopic pregnancy, stillbirth, and therapeutic abortion. Informed consent was obtained from all study participants, and 6–10 ml peripheral blood were drawn from each individual. All contacts with study participants (i.e., selection, venipuncture, and specimen numbering) were made by one individual, who was not involved in cytogenetic analysis.

Cytogenetic Analysis

Each sample was sequentially numbered and delivered to the laboratory, with no other identifying information. Specimens were prepared for cytogenetic study by routine methods (Moorhead et al. 1960). Chromosome spreads were analyzed by silver staining in a two-step procedure according to the protocol of Howell and Black (1978). Four drops of 50% $AgNO_3$ were pipetted onto the slide, and a coverslip was placed over the slide. The slide was placed under a high-intensity lamp until the $AgNO_3$ crystallized, and the coverslip was removed by rinsing with running water. This was followed by addition of two drops of 3% formalin and two drops of ammoniacal silver (4 g silver nitrate dissolved in 5 ml sterile distilled water and 7.5 ml ammonium hydroxide). A second coverslip was applied immediately, and the reaction was visually monitored at the microscope. When the cells turned a deep yellow and the NORs appeared black, the silver solution was washed off. Identification of individual chromosomes was accomplished by trypsin-Giemsa banding following silver staining.

Parental Origin of Nondisjunction

Determination of parental origin of nondisjunction was performed using chromosomal heteromorphisms and DNA polymorphisms. Q-banding was carried out by staining in 0.5% quinacrine dihydrochloride for 8 min, followed by rinsing with water and incubation for 2 min in MacIlvaine's buffer (pH 5.6) (Uchida and Lin 1974). Preparations were also made for trypsin-Giemsa G-banding by a standard protocol, and satellite heteromorphisms were analyzed. Four families were studied using chromosomal polymorphisms.

Three families were studied by analysis of segregation of RFLPs. Six probes localized to chromosome 21 were tested: D21S13, D21S15, D21S16, D21S17, D21S19 (provided by S. Latt), and p21-4U (D. Kurnit, personal communication). DNA from peripheral blood of the family members was extracted, electrophoresed, and transferred to Gene Screen Plus membrane according to the New England Nuclear (NEN) protocol. Chromosome 21–specific probes were labeled by random oligonucleotide priming (Feinberg and Vogelstein 1984).

NOR Classification

The subjective nature of the classification of chromosomal variants observable after silver staining led us to develop a classification system for the size and configuration of the silver-stained region over each of the acrocentric chromosomes. The NOR of each acrocentric chromosome was assigned a score from 0 to 5 on the basis of the size of the silver-stained region. Criteria for each of the scores are listed in table 1.

Fifteen metaphase spreads from each study participant were examined, and each individual was evaluated by two investigators. One investigator scored 10 cells, and the second investigator scored five. Photographs were taken of all cells containing an acrocentric chromosome with a score of 3, 4, or 5. Each individual was assigned to one of four classes on the basis of the score of the acrocentric chromosome with the largest NOR (providing that this configuration was seen in at least five of the 15 cells examined). Throughout the

Table 1

Criteria for Scoring Silver-stained Acrocentric Chromosomes

Initial Score	Staining Characteristics	Class
0	Absent Stain	IV
1	Small stained NOR, size of double minute	IV
2	NOR size of short arm of chromosome 18 in the same metaphase	IV
3	NOR size of short arm of chromosome 20 in the same metaphase	III
4	Two foci of silver not separated by a clear area, demonstrating a constriction, suggesting two areas of stain close together	II
5	Two distinct foci of silver staining with a space between	I

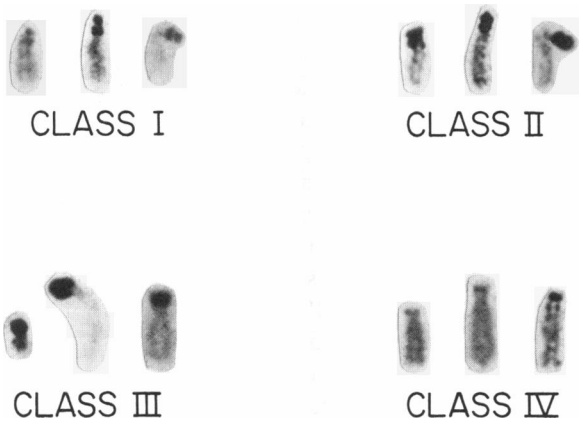

Figure 1 Examples of the four classes of silver staining. A chromosome had a class I pattern if there were two foci of silver and a space between them; it had a class II pattern if there were two foci, not separated. Classes III and IV had single regions of silver staining, with class III being larger than class IV.

study, we found that both investigators did agree on score assignments. In addition, pictures from three cells from each of a number of study participants were presented blindly to a third individual, who was asked to score each cell. Again there was agreement in all cases.

An individual was considered to be in class I if at least one acrocentric chromosome demonstrated two foci of silver staining with a space between them (in at least five of the 15 cells examined) (fig. 1). Class II contained individuals who had at least one acrocentric chromosome with two foci of silver, not separated by a clear area but demonstrating a constriction, suggesting two areas of silver stain close together. Class III contained individuals whose largest silver-stained acrocentric chromosome demonstrated a single large area of silver stain, approximately equal in size to the short arm of chromosome 20 in the same cell. Class IV consisted of individuals whose acrocentric chromosomes all demonstrated a single, small area of silver stain or no silver stain (fig. 1). Class I and class II NORs were considered to be equivalent to the "double NORs" described elsewhere (Jackson-Cook et al. 1985).

Variation in Silver Staining

From cell to cell within a given preparation, there is variation in the morphology of the silver stain over a particular acrocentric chromosome. This variation has been observed by numerous investigators studying silver-stained chromosome preparations (Starkman and Shaw 1967; Gigliani et al. 1972; Archidiacono et al. 1977; Bernstein et al. 1981; Jackson-Cook et al. 1985). Figure 2 demonstrates two adjacent metaphases from the same individual. The upper metaphase demonstrates a class I pattern of silver staining, while the lower metaphase demonstrates a class III pattern. In this study, an individual was assigned to the lowest numerical class applicable, providing that that category variant was identified in five of the 15 cells examined. In the example cited above, the individual was assigned to class I.

Results

Frequencies of NOR Variants

We have analyzed 108 samples from 29 case couples (58 individuals) and 25 control couples (50 individuals). Each individual was assigned to class I, II, III, or IV. Of the 108 individuals studied, four (3.7%) were found to have a class I NOR, 10 (9.26%) were found to have a class II NOR, 14 (13%) were found to have a class III NOR, and 80 (74%) had class IV NORs. The distribution of cases and controls among each of these classes is indicated in table 2. Seven (12%) of 58 case individuals had class I or II NORs, compared with seven (14%) of 50 control individuals. Class I and II NOR variants are equally distributed between D and G group chromosomes in cases and controls (for cases, there were four D and three G; for controls, there were four D and three G) (table 3).

Origin of Nondisjunction

Nondisjunction leading to trisomy 21 occurred in only one member of each couple. Therefore, we attempted to determine the parental origin of nondisjunction in all case couples with a class I or II NOR, to determine whether the parent in whom nondisjunction occurred also had the NOR variant.

Among the cases there were seven individuals (6, 19, 31, 32, 44, 50, and 100) who were found to have a class I or class II NOR variant. The family of individual 6 was lost to follow-up. The origin of nondisjunction leading to trisomy 21 could not be identified in families of cases 19 or 100, as all polymorphisms tested were uninformative.

The parental origin of nondisjunction could be determined for two families by using RFLP analysis. Figure 3 shows *Taq*I-digested DNA from the family of individuals 31 and 32 that was probed with D21S13. Thus, the father and mother were homozygotes for the 5.5-kb and 6.5-kb alleles, respectively, whereas the three normal heterozygous offspring showed hybridization

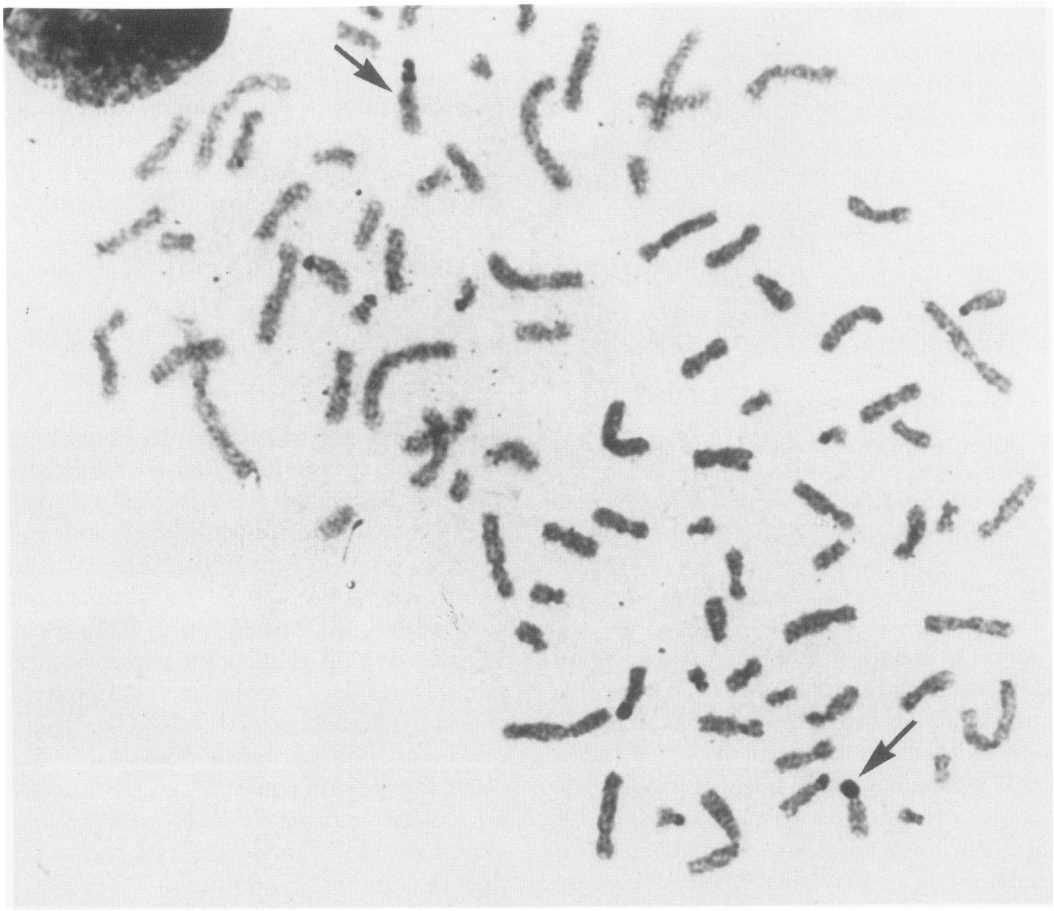

Figure 2 Example of the variation seen from metaphase to metaphase within a sample. two adjacent metaphases from a silver-stained slide are shown. The upper metaphase demonstrates a class I pattern, and the lower metaphase demonstrates a class II pattern.

to both alleles. The affected child showed a dosage difference, with a more intense signal for the 6.5-kb allele, which usually is the less intense of these two bands. This indicates maternal nondisjunction as the source of the extra chromosome 21. Similarly, DNA studies of case 50, using a probe for locus D21S19 and the enzyme *Msp*I, demonstrated greater hybridization for the maternally derived allele in the affected offspring. A maternal origin of nondisjunction was confirmed in this family by using fluorescent satellite heteromorphisms. The class II NOR variant was seen in the father.

In the family of case 44, G-banding heteromorphisms were informative. The father had long stalks on both chromosomes 21, and the mother had one long stalk and one short stalk. The child with trisomy 21 inherited two short stalks and one long stalk. Therefore, nondis-

Table 2

Distribution of Cases and Controls among the Four Classes of Silver-stained Chromosomes

	No. (%) of Cases by Class (ES)					
	I	II	I and II	III	IV	Total
Case	2 (3.4)	5 (8.6)	7 (12)	8 (13.8)	43 (74)	58
Control	2 (4.0)	5 (10)	7 (14)	6 (12)	37 (74)	50

Table 3

Comparison of Parent with Variant NOR and Parent in Whom Nondisjunction Occurred, in Cases with Class I or Class II NORs

Case no.	NOR Class	Chromosome with Variant NOR	Parent with Variant	Parent with Nondisjunction
6	I	22	Father	?[a]
19	II	14	Mother	?[b]
31	II	14	Mother and father	Mother
32	II	14	Mother and father	Mother
44	II	22	Father	Mother
50	II	13	Father	Mother
100	I	22	Father	?[b]

[a] Lost to follow-up.
[b] All polymorphisms tested were uninformative.

junction had to be of maternal origin, while the class II NOR variant was seen in the father (table 3).

We have demonstrated that individuals 32, 44, and 50 are not the parent in whom the nondisjunction event occurred. If a case is considered to be an individual in whom nondisjunction leading to trisomy 21 occurred, the number of "cases" with an NOR variant is reduced to a maximum of four (13.8%) of 29, compared with seven (14%) of 50 among controls (table 2).

Discussion

The short arms of the acrocentric chromosomes show a high degree of variation in size and staining pattern. As such, they have been investigated extensively in an attempt to correlate their heteromorphisms with specific diseases (Tjio et al. 1960; Dekaban et al. 1963; Hamerton et al. 1965; Starkman and Shaw 1967; Jackson-Cook et al. 1985). However, there is no proven relationship between acrocentric chromosomal variants and any disease or phenotype. In addition, the acrocentric short arms have been considered to be redundant, since they contain highly reiterated DNA sequences and because persons with Robertsonian translocations have no phenotypic abnormalities (Schmickel et al. 1985). A number of investigators have focused on the correlation between changes in the short arms of the acrocentric chromosomes and a predisposition to trisomy 21 (table 4). However, variations in this region of the genome have been reported in studies of normal individuals (Court-Brown et al. 1965; Hamerton et al. 1975).

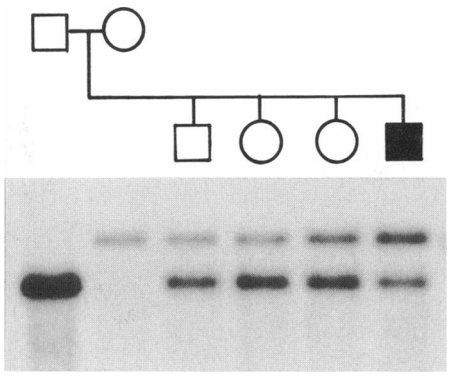

Figure 3 Southern blots of DNA, digested with *TaqI* and probed with D21S13, from the family of individuals 32 (father) and 31 (mother). The individual in lane 6 had trisomy 21 and demonstrates increased dosage of the maternal allele.

In a study reported by Jackson-Cook et al. (1985) a variation of silver staining was reported to be associated with a significantly increased risk (as high as 20-fold) for having offspring with trisomy 21. Our study was designed to test their hypothesis. Using a blinded, case-control study design, we found that there is not an association between having an offspring with trisomy 21 and silver-staining variants.

In the previous study (Jackson-Cook et al. 1985) "double NOR variants" were observed in 15 (10%) of 150 individuals studied. In our study, the double NOR variants (class I and class II) were observed in 14 (13%) of 108 individuals. In another population study of 200 individuals, 17 (8.5%) were found, by silver staining, to have tandem duplications of the NOR region (Chambers and Priest 1986). Hassold et al. (1987) studied NORs both of parents of 23 spontaneous abortuses trisomic for an acrocentric chromosome and of 55 control couples with either chromosomally normal spontaneous abortions, abortuses with sex chromosome monosomies, or trisomies for nonacrocentric chromo-

Table 4

Surveys in Which Enlarged Short Arms of Acrocentric Chromosomes Were Studied

Investigator	*N* (population)	Proportion (%) of Atypical Acrocentrics
Dekaban et al. (1963)	14 (+21)	3/14 (21.4)
Hamerton et al. (1965)	144 (+21)	2/144 (1.4)
	70 (parents)	1/70 (1.4)
Court-Brown et al. (1965)	438 (controls)	7/438 (1.5)
Edgren et al. (1966)	73 (+21)	5/73 (6.8)
Starkman and Shaw (1967)	40 (+21)	8/40 (20)
	40 (controls)	3/40 (7.5)
Sands (1969)	232 (+21)	8/232 (3.4)
	142 (controls)	4/142 (2.8)
Hamerton et al. (1975)	14,069 (newborns)	125/14,069 (.89)
Jackson-Cook et al. (1985)	41 (parent of +21 in whom nondisjunction occurred)	13/41 (32)
	50 Controls	0/50
Hassold et al. (1987)	44 Parents of abortuses with acrocentric trisomy	0/44
	106 Controls	0/106

somes, a total of 150 individuals. They were unable to detect a single "double NOR." However, their criteria were such that they scored only chromosomes with two separate silver-positive regions. These would be equivalent to our class I variants seen in four (3.8%) of 108 individuals, i.e., in two of 58 cases and in two of 50 control individuals.

The differences observed in these studies may point to important differences in methodology or in interpretation of NOR variants. In the present study, we attempted to establish strict criteria for classification of NOR variants (separate silver-positive regions, [class I], dumbell-shaped regions [class II], large single regions [class III]) and then to have all samples scored by cytogeneticists blind to the status of the case, permitting us to detect any differences between the two groups.

Our findings on the frequency of the NOR variants in the normal population were different from those of Jackson-Cook et al. (1985). We found class I and class II variants to be equally prevalent among cases and controls (four of 29 cases, compared with seven of 50 controls). The sample size of this study is sufficient to detect a 4.5-fold increased risk for trisomy 21 associated with these NOR variants. This calculation is based on a case-control design, a power of 80%, and a one-sided significance level of .05. In contrast, in the Jackson-Cook et al. (1985) study, double NOR variants were found in 13 of 41 parents of children with trisomy 21 in whom nondisjunction occurred, in one of 41 spouses not responsible for nondisjunction, and not at all in the control population. Their data suggested that the presence of a double NOR variant may be associated with a 20-fold increased risk for having a child with trisomy 21. These striking differences may be the result of methodological differences between the two studies. Our study was accomplished in a blinded fashion, and we feel that the technical variation seen in the scoring of silver-stain variants necessitates this approach.

The silver-staining technique specifically stains the NORs on the acrocentric chromosomes 13, 14, 15, 21, and 22 (Goodpasture and Bloom 1975). These regions are the sites of the tandemly repeated ribosomal genes, present in about 40 copies/acrocentric chromosome (Schmickel 1973). There is variation in the number of genes per chromosome, as determined by in situ quantitation (Evans et al. 1974). The number of genes and the transcriptional activity of the genes account for the cytologic variation observed on silver staining (Evans et al. 1974; Miller et al. 1976; Schmickel and Knoller 1977). Double NORs most likely arise from unequal homologous recombination within the ribosomal genes (Schmickel et al. 1985). It has been proposed that the NORs may play a part in the origin of nondisjunction leading to trisomy 21. In the nucleolus, acrocentric chromosomes associate, and it has been proposed that this might interfere with disjunction of homologous chromosomes in meiosis (Polani et al. 1960). However, our data suggest that cytologic variations in size and configu-

ration of the NOR regions do not correlate with a tendency for nondisjunction events of the acrocentric chromosomes.

In conclusion, we have developed a reproducible system for the classification of silver-stained NOR variations on the basis of their size and configuration. Using this classification system, we have determined that the presence of a class I or class II NOR is not significantly increased in families with Down syndrome. Thus, our results do not support the use of NOR studies for predicting couples at increased risk for having a child with trisomy 21.

An understanding of the underlying molecular structure of class I and class II NORs may provide additional information to support our conclusions. Such findings may permit us to determine the origins of double NORs and to clarify their relationship to meiosis. Variations of the staining characteristics of the short arms of the acrocentric chromosomes remain an interesting phenomenon and provide an excellent marker for determining parental origin of the chromosomes involved in nondisjunction. However, acrocentric chromosome markers such as the double NOR do not appear to contribute to the etiology of nondisjunction.

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