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Nonrandom X-Chromosome Inactivation in Hemopoietic Cells from Carriers of Dyskeratosis Congenita

To the Editor:

Dyskeratosis congenita (DC) is a rare inherited ectodermal dysplasia characterized by dermatologic manifestations and nail dystrophy. Bone-marrow failure has been reported to occur in ~50% of cases (Dokal 1996), and in some patients symptoms related to aplastic anemia may precede the diagnosis of DC (Forni et al. 1993).

The gene for the X-linked recessive form of DC has been assigned to Xq28 by X chromosome–specific RFLPs (Connor et al. 1986; Arngrimsson et al. 1993; Knight et al. 1996), but the primary defect responsible for the disease is still unknown. Existing methods for carrier detection therefore rely on analysis of genetic linkage and are limited both by the availability of samples from adequate pedigrees and by the degree of polymorphism of closely linked markers within a kindred.

More recently, in vitro clonogenic assays, as well as long-term bone marrow–culture studies have suggested that symptoms of aplastic anemia in DC might be due to a defect at the level of the hematopoietic stem cell (Marsh et al. 1992). For this reason, we hypothesized that, at least in some DC families, the selective pressure in the heterozygote might be strong enough to determine negative selection of progenitors bearing the mutant allele, resulting in extreme skewing of X-chromosome inactivation in cells of hemopoietic descent. Selection against cells bearing the mutant allele has been demonstrated in heterozygotes for several X-linked disorders (Belmont 1996), especially immunodeficiencies (Gealy et al. 1980; Conley et al. 1986), and in such families assessment of randomness of X-chromosome inactivation has been successfully used to test for carrier status (Puck et al. 1987).

We examined two kindreds in which the probands had been found to have DC after severe symptoms of bone-marrow failure had developed, in order to evaluate X–inactivation mosaicism in hemopoietic cells from obligate carriers and other female members of the family. Criteria for diagnosis of DC were the findings of nail dystrophy, reticulate hyperpigmented skin lesions, oral leukoplakia, and severe pancytopenia in the probands. Both examination of the pedigrees (figs. 1 and 2) and linkage analysis (Knight et al. 1996) were consistent with an X-linked recessive disorder in both families.

Whole blood leukocytes (WBC), granulocytes (PMN), and mononuclear cells (MNC) were isolated according to standard methods; the purity of hemopoietic cell populations was evaluated by direct examination of cytocentrifuged slides stained with May-Grünwald-Giemsa.

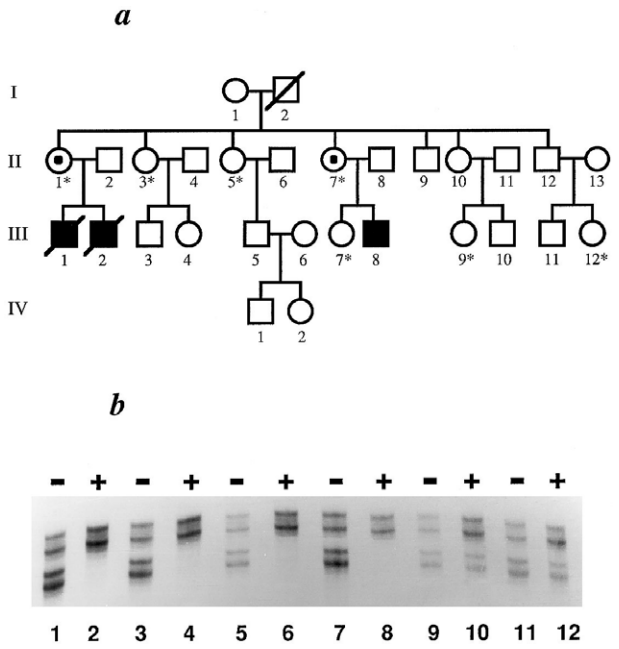

Figure 1 Analysis of family A. *a,* Pedigree (asterisks indicate women tested for X-chromosome inactivation). *b,* AR-methylation PCR assay. The DNA was predigested with (lanes denoted "+") and without (lanes denoted "−") the methylation-sensitive enzyme *Hpa*II; each allele is represented by two bands. Lanes 1 and 2, PMN from II-1. Lanes 3 and 4, MNC from subject II-1. Lanes 5 and 6, PMN from II-7. Lanes 7 and 8, MNC from II-7. Lanes 9 and 10, PMN from III-7. Lanes 11 and 12, MNC from III-7. Absence of the lower allele in the "+" lanes indicates nonrandom X-chromosome inactivation.

DNA was extracted and purified according to routine procedures. X–inactivation analysis was performed by taking advantage of the favorable characteristics of heterozygosity at the X-linked human androgen receptor (AR) locus—namely, the high percentage of informative subjects, the advantage of a PCR assay, and the consistent pattern of methylation of *Hpa*II and *Hha*I sites within a highly polymorphic CAG repeat in the coding region of the first exon of the AR gene (Allen et al. 1992). The details of the PCR analysis, run in the presence of a radioactive nucleotide, have been described elsewhere (Willman et al. 1994; Ferraris et al. 1997). Visual assessment of relative hybridization intensities by inspection of the autoradiographs was confirmed by quantitation of the two AR alleles, by comparison of radioactive intensity of the bands by means of a densitometer. All assays were performed in duplicate, with the assayer blinded to the identity of the samples.

Sixteen women, seven of them from family A and nine of them from family B, were investigated after informed consent was obtained (table 1). The DNA of all subjects was extracted from WBC. In addition, purified fractions of PMN and MNC were isolated from all obligate carriers (two from family A and two from family B) and

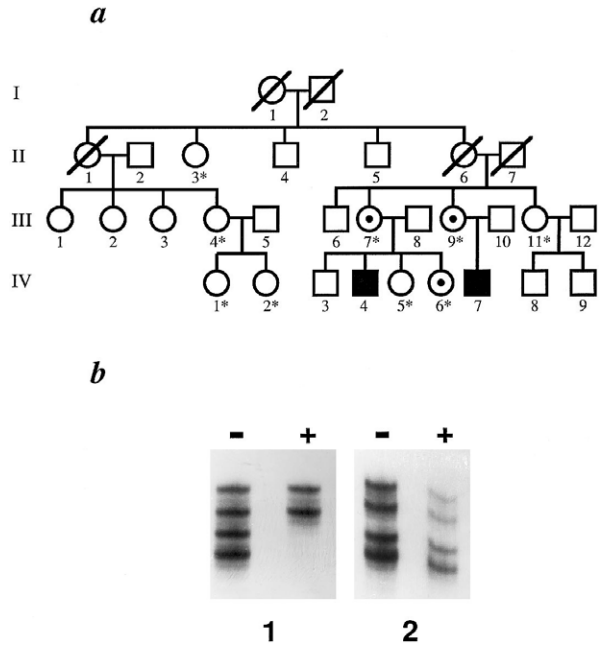

Figure 2 Analysis of family B. *a*, Pedigree (asterisks [*] indicate subjects tested for X-chromosome inactivation). *b*, Representative PCR AR assays (details of test are as in fig. 1). Panel 1 shows selective use of a single X chromosome as the active one in PMN cells from III-7; panel 2 shows random X-chromosome inactivation in PMN from IV-5.

from four other females (one from family A and three from family B) of unknown carrier status (table 1).

All women were informative at the AR locus, thus confirming both reports by others (Naumova et al. 1996) and our own experience (164 [89%] heterozygous women, of 185 screened so far) of the very high percentage of polymorphism with this marker. Artificial mixtures made of DNA from control women homozygous for alleles with different numbers of CAG repeats in the AR gene showed that identification of the minor component was unequivocal when it constituted $\geq 5\%$ of the total (Ferraris et al. 1997; A. M. Ferraris, unpublished data). In our control set of AR heterozygotes, the allelic ratio after *Hpa*II digestion was found to be consistent with random X-chromosome inactivation in $>90\%$ (49 of 53) of the women tested. Therefore, if we consider those unrelated women whom we sampled specifically as a control group, only 7.5% of them had $>95\%$ of their blood cells with the same X chromosome active. The results of our analysis of X–inactivation patterns in normal females are in full accordance with those reported by other investigators (Busque et al. 1996; Naumova et al. 1996). Consequently, it can be safely stated that excessive skewing, defined as $\geq 95\%$ expression of one X-linked allele, is an infrequent event in healthy women without a family history of a genetic disorder.

In family A the two obligate carriers were found to have nonrandom X-chromosome inactivation in their PMN and MNC, whereas all other subjects tested, including the sister of the propositus, exhibited a balanced pattern of X-chromosome inactivation (table 1). A sample of the results obtained with the PCR amplification of the AR gene from this kindred is shown in the lower part of figure 1.

In the second family, the mothers of the two affected boys also expressed nonrandom use of the X chromosome in their hemopoietic cells; of the two sisters of a proband, one was found to have nonrandom X-chromosome inactivation and the other exhibited a random pattern of X-chromosome inactivation (table 1). In the lower half of figure 2 are compared the results of X–inactivation analysis performed on PMN from one obligate carrier, who was nonrandomly inactivated, and the results obtained on one of her daughters, who had random inactivation of the X chromosome. Although only a few examples are shown here, these results are representative of our findings on other females studied, both from within these two pedigrees and from other sources (Ferraris et al. 1997).

Subject IV-6 from this pedigree is the only individual from the two families who has a skewed X–inactivation pattern and a prior probability of being a carrier of .5. After being tested, she might be correctly identified as a carrier, in which case the conditional probability is 1.0, but she might simply be skewed on a chance basis, with a conditional probability of .075, since, on the basis of

Table 1

X-Chromosome Inactivation in Women from Two DC Families

Family and Subject (Age [years])	Cell Populations Tested	Pattern of X Inactivation[a]
A:		
II-1 (51)	WBC, PMN, MNC	Nonrandom
II-3 (48)	WBC	Random
II-5 (46)	WBC	Random
II-7 (44)	WBC, PMN, MNC	Nonrandom
III-7 (23)	WBC, PMN, MNC	Random
III-9 (20)	WBC	Random
III-12 (16)	WBC	Random
B:		
II-3 (65)	WBC	Random
III-4 (47)	WBC	Random
III-7 (40)	WBC, PMN, MNC	Nonrandom
III-9 (37)	WBC, PMN, MNC	Nonrandom
III-11 (35)	WBC, PMN, MNC	Random
IV-1 (28)	WBC	Random
IV-2 (27)	WBC	Random
IV-5 (17	WBC, PMN, MNC	Random
IV-6 (8)	WBC, PMN, MNC	Nonrandom

[a] Nonrandom X inactivation is >90:10.

our data, 7.5% of normal women have >95% of their blood cells with the same X chromosome active. The joint probability of being correctly identified as a carrier would then be .5 (.5 × 1), whereas the joint probability of being a skewed noncarrier would be .0375 (.5 × .075). Therefore, the probability of being correctly identified as a carrier would be .93 [.5/(.5 + .0375)]. This constraint should be explained carefully to any prospective consultand in a similar situation.

Our conclusion is that obligate-carrier status, as determined by disease segregation, was correlated with a pattern of nonrandom X-chromosome inactivation in every case. Moreover, selective inactivation of the X chromosome occurs within several hemopoietic cell populations in obligate carriers of X-linked DC. Taking into consideration that both a preliminary report communicated in abstract form (Langlois et al. 1993) and a recently published paper on a DC family (Devriendt et al. 1997) had reached the same conclusion, we believe that the data from three separate studies do strongly support the idea that DC carrier status is a cause of skewed X-chromosome inactivation in blood cells.

Whenever a diagnosis of X-linked DC is made, identification of carrier status in female relatives is important for genetic counseling. A small error rate cannot be avoided when linked probes alone are used for carrier detection, and it is not possible to identify new mutations by means of linked DNA markers. Moreover, there will always be boys without a family history of DC for whom it is not clear whether they have inherited an X-linked or autosomal recessive form of the disease. For the families of these boys, analysis of X–inactivation patterns in maternal hemopoietic cells will help clarify future risks for members of the family. For example, for a singleton case of DC, one would estimate the prior probability of the mother being a carrier. A conventional approach would be to set the prior probability at .67 ($^2/_3$), but, because of higher mutational rate in the paternal germ line, the real probability may approach .9. With the more conservative figure and a similar analysis, the finding of skewed X-chromosome inactivation in the mother of a singleton case would shift the probability of being a carrier to .96.

For these reasons, the use of the AR assay for methylation analysis, while not diagnostic, promises to be beneficial with regard to the ability to provide genetic counseling, by altering the estimate of risk of recurrence in DC, until testing of specific genes becomes available. The technical characteristics of the AR assay are advantageous in this respect; since only a small amount of DNA is necessary for initial testing, the whole procedure can yield definitive results in <1 wk and is relatively cheap, especially when compared with currently used techniques of genetic linkage and mapping.

The present report can then be characterized as a confirmatory comprehensive investigation of the pattern of X-chromosome inactivation in various hemopoietic cells of women from two large DC families. We have observed selective inactivation phenomena in the PMN and MNC of obligate carriers, implying that the DC gene defect is expressed in each of these cell populations. To the extent that tissue-specific selective inactivation reflects the primary cell target(s) of the gene defect, the analysis of X–inactivation pattern should help to determine the cellular and molecular bases for DC, as has been the case for other X-linked disorders, thereby providing an additional step toward identification of the gene(s) involved.

Anna Maria Ferraris,[1] Gian Luca Forni,[2] Rosa Mangerini,[1] and Gian Franco Gaetani[1]
[1]*Dipartimento di Oncologia Clinica e Sperimentale, Università di Genova and Istituto Nazionale per la Ricerca sul Cancro, and* [2]*Divisione di Pediatria, Ospedali Galliera, Genoa*

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Address for correspondence and reprints: Dr. Anna Maria Ferraris, Ematologia Oncologica, IST, Largo Rosanna Benzi, 10, 16132 Genova, Italy.

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Is There an Abnormal Phenotype Associated with Maternal Isodisomy for Chromosome 2 in the Presence of Two Isochromosomes?

To the Editor:

We read with great interest the recent report by Bernasconi et al. (1996) describing a case of maternal isodisomy 2 due to the de novo inheritance of two isochromosomes for chromosome 2. We recently identified an additional case of maternal isodisomy 2 also caused by the de novo inheritance of two isochromosomes for chromosome 2. However, in our case, the child has some significant features that are in common with recently reported cases of maternal disomy 2 (Bernard et al. 1995; Harrison et al. 1995; Webb et al. 1996). Our case was the 785-g male product of a 31-wk gestation to a 28-year-old G2P0 mother. Delivery was via cesarean section because of pregnancy-induced hypertension, severe intrauterine growth retardation (IUGR), and marked oligohydramnios. Apgar scores were 8 and 9 at 1 and 5 min, respectively. The birth weight and length (33 cm) were significantly below the 3d percentile (10th percentile for 26-wk gestation). The baby was stable on room air until 13 d of age, when he had an acute deterioration, with low oxygen saturation requiring hood oxygen of 40%–80% for several days. His oxygen requirements dropped into the 23% range by 3 wk of age, and he continued to require oxygen until 12 mo of age. At 2 mo of age a chest x-ray showed bilateral interstitial densities resembling bronchopulmonary dysplasia. At birth, a perineal hypospadias was evident and was subsequently repaired. Bilateral preauricular ear pits and significant pectus carinatum, without other dysmorphic features, were noted during an examination at 8 years of age. At this time, his height was 116 cm (<2 SD for age, corrected for midparental height). Chromosome analyses revealed an apparently nonmosaic 46,XY,i(2)(p10),i(2)(q10) karyotype (fig. 1). Molecular studies using seven dinucleotide-repeat polymorphisms for chromosome 2 revealed inheritance of only maternal alleles and failure of inheritance of any paternal alleles in the child (fig. 2). Additionally, the mother was heterozygous for all markers tested, and the child inherited only one allele for each marker, resulting in homozygosity for both chromosome arms, consistent with isochromosomes. The finding of true isochromosomes in our case and in that reported by Bernasconi et al. (1996) most likely formed through misdivision of the centromere in a monosomy-2 conceptus. Other possibilities, as discussed elsewhere (Bernasconi et al. 1996), also exist.

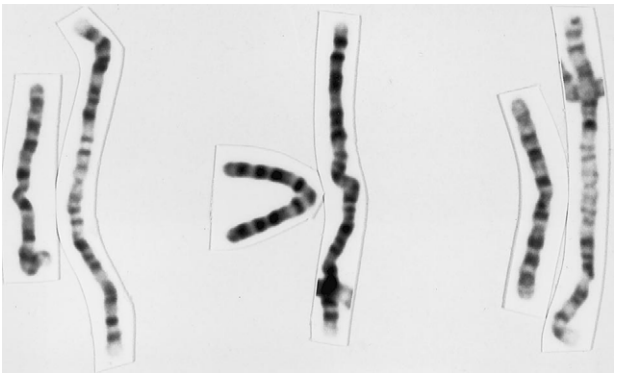

Figure 1 Three partial G-banded metaphases showing the isochromosome for 2p (*left*) and the isochromosome for 2q (*right*) in the proband.

In sharp contrast to the case presented by Bernasconi et al., in which there were no apparent phenotypic ab-