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Detection of Deletions and Cryptic Translocations in Miller-Dieker Syndrome by In Situ Hybridization

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Summary

Fluorescence in situ hybridization (FISH) using two cosmid probes (41A and P13) from the Miller-Dieker syndrome (MDS) critical region in 17p13.3 was performed in a blinded comparison of three MDS patients with submicroscopic deletions and in four normal relatives used as controls. The controls showed both chromosome 17 homologues labeled in 85%–95% of cells, while each patient showed only one homologue labeled in 75%–80% of cells. Two MDS patients with cryptic translocations were also studied. In one case, a patient and her mother had the same der(17) (p+), but the reciprocal product of the translocation could not be identified in the mother by G-banding (i.e., it was a "half-cryptic" translocation). FISH revealed a 3q;17p translocation. The other case involved a patient with apparently normal karyotype. Because a large molecular deletion was found, a translocation involving two G-negative telomeres (i.e., a "full-cryptic" translocation) was postulated. FISH studies on her father and normal brother showed an 8q;17p translocation. These studies demonstrate that in situ hybridization is an efficient method for deletion detection in Miller-Dieker syndrome. More important, parental studies by FISH on patients demonstrating molecular deletions and a normal karyotype may identify cryptic translocation events, which cannot be detected by other molecular genetic strategies. Similar in situ strategies for deletion detection can be developed for other microdeletion syndromes, such as Prader-Willi/Angelman syndrome or DiGeorge syndrome.

Introduction

Miller-Dieker syndrome (MDS) is a rare malformation syndrome manifested by type I lissencephaly (smooth brain) and a characteristic facies (Dobyns et al. 1991). Lissencephaly results from an arrest of neuronal migration at about 10–14 wk of embryonic development. The characteristic facies consists of a prominent forehead, bitemporal hollowing, short nose with upturned nares, protuberant upper lip, thin vermillion border, and small jaw.

MDS is associated with a microdeletion of chromosome 17p13.3, which is visible by high-resolution cytogenetic methods in about 50% of cases (Dobyns et al. 1991). In patients without a visible deletion, large molecular deletions can be detected by RFLP analysis using several anonymous DNA markers located within the MDS critical region (Schwartz et al. 1988; van-Tuinen et al. 1988; Ledbetter et al. 1989). However, RFLP studies are not always fully informative. In the past, we have studied uninformative patients by segregating the maternal and paternal homologues of chromosome 17 into different somatic cell hybrids. However, the construction of somatic cell hybrids is labor intensive and is not practical as a routine diagnostic tool.

Recent improvements in nonisotopic methods for in situ hybridization have greatly improved the speed and accuracy of this technique for gene mapping (Kievits et al. 1990; Rowley et al. 1990), as well as for the identification of chromosomal rearrangements. To date, the power of in situ hybridization in clinical cytogenetics has been demonstrated for the detection of specific translocations, such as the 9;22 translocation

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in chronic myelogenous leukemia (Arnoldus et al. 1990; Tkachuk et al. 1990), the inversion chromosome 16 in acute nonlymphocytic leukemia (Dauwerse et al. 1990), and detection of deletions in hereditary spherocytosis (Lux et al. 1990) and Duchenne muscular dystrophy (Ried et al. 1990). Here we show that in situ hybridization is a rapid and highly efficient method for the detection of submicroscopic deletions in MDS patients and for the identification of cryptic translocation events. This approach may be extended to other microdeletion syndromes and has the potential to supplement or replace high-resolution cytogenetic methods for investigating these disorders.

Subjects, Material, and Methods

Patients and Controls

A blinded analysis of three MDS patients and four of their normal relatives used as controls was performed to determine the efficiency of in situ hybridization for deletion detection. These three patients—MDS-13, MDS-19 and MDS-22—have been reported elsewhere (Dobyns et al. 1991). In brief, all three had characteristic MDS phenotypes including type I lissencephaly and typical craniofacial abnormalities but did not have cytogenetically detectable deletions of 17p13.3. Molecular deletions within band 17p13.3 were previously demonstrated by somatic cell hybrid analysis of MDS-13 and MDS-19, by using probes YNZ22 and YNH37 (Dobyns et al. 1991). MDS-22 was uninformative by RFLP studies, and somatic cell hybrid studies have not been performed. A deletion was suspected, however, since the patient showed a single allele of reduced intensity for YNZ22 and YNH37. Controls included the parents of MDS-19 (coded as subjects 1 and 2), the mother of MDS-22 (subject 5), and the father of MDS-9 (subject 3).

In addition, two MDS families with known or suspected translocations were studied. MDS-18 was the first child of unrelated parents. She had typical features of MDS, and cytogenetic analysis showed an abnormal G-positive band on 17p (der(17) (p +)). Her mother had the same der(17) (p +), but high-resolution chromosome analysis failed to reveal the reciprocal product. We will refer to this as a "half-cryptic" translocation. Molecular analysis using RFLPs confirmed a maternally derived deletion, including probes YNZ22, YNH37, and 144D6 (Dobyns et al. 1991).

MDS-6 was the second child of unrelated parents, who also had one normal child and three previous spontaneous abortions. She had typical features of MDS, and results of high-resolution chromosome analysis were normal (van Tuinen et al. 1988; Dobyns et al. 1991). Molecular analysis of somatic cell hybrids containing the paternal chromosome 17 showed a large DNA deletion, including probes YNZ22, YNH37, and 144-D6 (Ledbetter et al. 1989). These results were somewhat discordant, as all other patients deleted for these three probes had visible cytogenetic deletions or translocations. Because this patient had two apparently normal chromosomes 17, the hypothesis was entertained that she represented an unbalanced, cryptic translocation involving two G-negative telomeres (i.e., a "full-cryptic" translocation).

Chromosome Preparations

Epstein-Barr virus–transformed lymphoblastoid cell lines from patients and from controls were used for cytogenetic preparations by routine methods. Air-dried slides were prepared the day prior to hybridization. For the blinded analysis, slides were numerically coded, and microscopic analysis was performed without knowledge of patient status.

Probes

Cosmids P13 and 41A have been described elsewhere (Ledbetter et al. 1989). Cosmid 41A (36 kb) contains probe YNZ22 and therefore maps within the common region of overlap for all MDS deletions. Cosmid P13 (35 kb) is also in the MDS critical region, and it overlaps cosmid 41A by less than 5 kb. Probes were labeled with biotin by nick translation (Tropix). Cosmids P13 (150 ng) and 41A (60 ng) were ethanol precipitated with 1.5 μg human placental DNA and 15 ng biotinylated chromosome 17 alpha-satellite probe (Oncor). The precipitate was resuspended in hybridization solution, which consisted of 50% formamide, 2 × SSC (pH 7.0), and 10% dextran sulfate. This gave a final concentration of 5 ng P13/μl, 2 ng 41A/μl, 500 ng placental DNA/μl, and 0.5 ng alpha-satellite DNA/μl. After being denatured for 5 min at 70°C, the DNA was allowed to partially anneal at 37°C for 3 h to compete out the repetitive sequences in the cosmid probes (Litt and White 1985; Lichter et al. 1990). Cohybridization with the alpha-satellite probe was performed in order to unambiguously identify chromosome 17.

In Situ Hybridization

In situ hybridization and detection was performed using the Chromosome In Situ Kit (Oncor) with slight

modifications. In brief, slides were treated with RNase in 2 × SSC at 37°C for 1 h, were washed in 2 × SSC, and were dehydrated in ethanol. After air-drying, slides were immersed in 70% formamide in 2 × SSC at 70°C for 2 min, quickly were dehydrated with four rinses (70%, 80%, 90%, and 100%) of cold ethanol, and were air-dried. Thirty microliters hybridization mixture was applied under a 24 × 50-mm coverslip. Slides were placed in a moist chamber for 16 h at 37°C. Slides were washed at 45°C in 50% formamide and 1 × SSC for 20 min, in 1 × SSC for 10 min, and in 0.1 × SSC for 5 min, and the final wash was in 0.1 × SSC at room temperature for 5 min. These wash conditions are at a higher stringency than those recommended in the Oncor kit, in order to reduce the background seen when using complex probes such as cosmids, large-insert phage clones, or yeast artificial chromosome (YAC) clones. The slides were immersed in 1 × PBD (Oncor) for 5 min.

After 20 min incubation at 37°C with fluorescein-labeled avidin (FITC), the signal was amplified with one additional layer of anti-avidin antibody. Slides were mounted in antifade medium containing propidium iodide as a chromosomal counterstain. Analysis of 20 metaphases was done on each coded sample, by using a Zeiss Axiophot microscope and filter combination 487709 (excitation at 450–490 nm). Color photographs were taken with Kodak Ektachrome 400 film.

G-Banding after Hybridization

After the fluorescent in situ metaphases were photographed, slides were destained with two changes of distilled water and were air-dried. G-banding was performed by Wright's-Giemsa staining in borate buffer (Cannizzaro and Emanuel 1984). Slides were pretreated by immersion for 5 min in borate buffer (50 mM Na_2SO_4, 2.5 mM $Na_2B_4O_7$; pH 9.2) prewarmed to 37°C in a water bath. Slides were submerged in a Petri dish containing a mixture of 3 parts borate buffer (pH 9.2) to 1 part Wright's-Giemsa stock solution for 6 min at 37°C.

Results

MDS versus Normal Controls

A positive fluorescence signal for the chromosome 17 alpha satellite was seen at the centromere region of both chromosome 17s in every metaphase analyzed. For the cosmid probes, a positive signal was defined as a fluorescence signal present on both chromatids of a chromosome (fig. 1, *top two panels*). This strict criterion eliminated almost all artifactual background signals and only slightly decreased the frequency of signals on 17p.

Of the seven coded samples, four were correctly identified as the normal controls and three were correctly diagnosed as the MDS-deletion patients (table 1). The four normal controls showed both chromosome 17 homologues to be labeled in 85%–95% of cells analyzed (fig. 1, *top two panels*) and one chromosome 17 to be labeled in the remaining 5%–15%. In the three MDS-deletion patients, no cells showed both homologues labeled, 75%–80% of cells showed one positive chromosome 17, and 20%–25% of cells showed no label on either chromosome 17. Examples for patients MDS-13 (fig. 1, *lower left*) and MDS-19 (fig. 1, *lower right*) are shown. It should be noted that these fluorescence in situ results were the first demonstration of a deletion in patient MDS-22, who was uninformative by previous RFLP analysis.

Detection of Cryptic Translocations

MDS-18, who had a der(17) (p+) karyotype and molecular deletion by RFLP studies, showed one positive signal on chromosome 17 in 90% of her cells and no signal on the remaining 10% (not shown). Her mother, who also had the der(17) (p+) karyotype, showed a signal on only one chromosome 17 but a second signal on the distal long arm of an A-group chromosome in approximately 80% of cells analyzed (fig. 2, *left*). G-banding analysis showed the positive signal to be on 3q, indicating a 3q;17p balanced translocation (fig. 2, *right*). Review of the G-banded karyotype of the mother failed to reveal any consistent alteration in the banding pattern of 3q (fig. 3). This suggests that the breakpoint occurred within the G-positive band q28 but that the reduction in size of q28 is not easily visualized on the derivative chromosome 3. The small G-positive piece translocated to 17p telomere, however, is detectable, since it makes that region much darker than normal. Her karyotype is thus designated 46,XX,t(3;17) (q28;p13.1).

MDS-6, who had normal chromosome 17s but a large molecular deletion by RFLP studies, showed one positive signal on chromosome 17 in 95% of her cells and no signal on the remaining 5% (not shown). Her father, who transmitted the deleted chromosome 17 to his daughter, showed a signal on only one chromosome 17 but a second signal on the distal long arm of a C-group chromosome in approximately 75% of cells

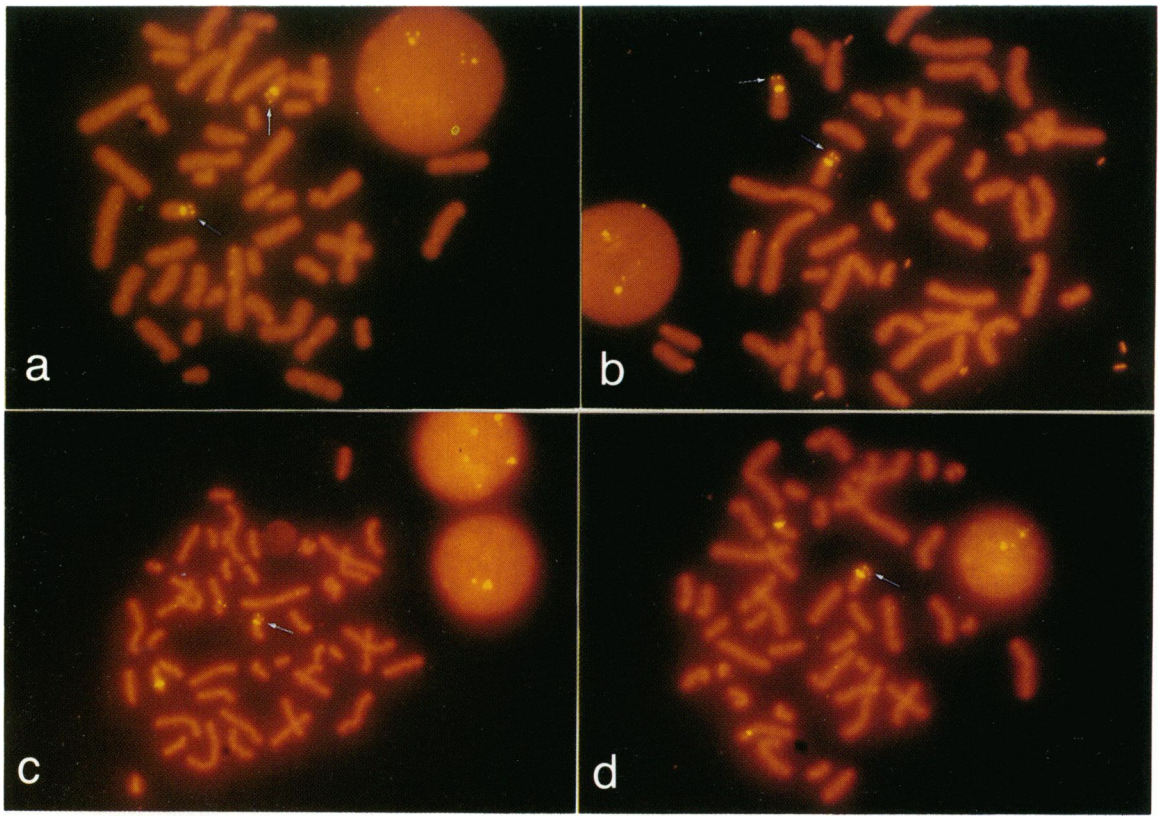

Figure 1 Examples of FISH to metaphases from two normal controls (*top two panels*) and from two MDS patients (*bottom two panels*). A single bright fluorescence signal is seen at the centromere of each chromosome 17 homologue, corresponding to the chromosome 17 alpha-satellite probe. Arrows indicate cosmid signals on both chromatids of distal 17p on both homologues of the two normal individuals but on only one chromosome 17 in MDS-13 (*bottom left*) and MDS-19 (*bottom right*). In MDS-13 the two background signals on a C-group chromosome are not scored as a positive, since one of the signals is not on the chromosome. In optimal interphase nuclei (*top left*), the two large alpha-satellite signals each associated with two small cosmid signals can be clearly distinguished.

Table 1

Frequency of Positive Signals for Cosmids P13 and 41A on Metaphase Chromosomes from MDS Patients and Normal Controls

	No. (%) of Positive Signals on				
Subject	Neither Chromosome 17	One Chromosome 17	Both Chromosomes 17	No. of Background Signals	Diagnosis
1	0	2 (10)	18 (90)	1	Normal
2	0	1 (5)	19 (95)	2	Normal
3	0	3 (15)	17 (85)	1	Normal
4	4 (20)	16 (80)	0	1	MDS-22
5	0	3 (15)	17 (85)	1	Normal
6	5 (25)	15 (75)	0	0	MDS-19
7	4 (20)	16 (80)	0	1	MDS-13

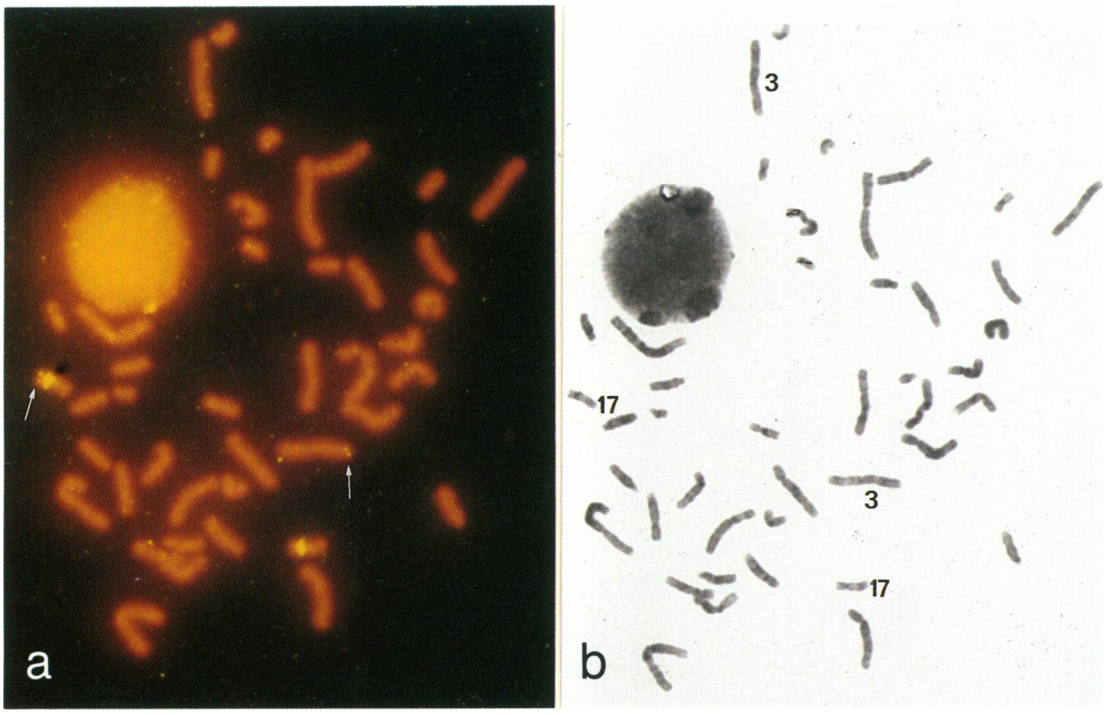

Figure 2 Sequential G-banding of mother of MDS-18, demonstrating 3q;17p translocation. FISH (*left*) showed only one cosmid signal on 17p, but a second signal was seen on the distal long arm of an A-group chromosome (*arrow*). G-banding (*right*) showed the signal to be on 3q.

analyzed (fig 4, *left*). G-banding analysis showed the positive signal to be on 8q, indicating an 8q;17p balanced translocation (fig. 4, *right*). Review of the G-banded karyotype of the father failed to reveal any alteration in the banding pattern of either chromosome 8 or chromosome 17. This suggests that the breakpoint occurred within the most distal G-negative band, q24.3. His karyotype is therefore 46,XY, t(8;17) (q24.3;p13.3). As expected, her mother showed a normal result, with signals on both chromosomes 17 in 90% of cells. The patient's phenotypically normal brother, however, was found to be a carrier of the 8q;17p translocation (fig. 5). Studies of the father's parents, I-1 and I-2, showed normal results, with two signals on chromosomes 17 in 85% and 80% of cells. This indicates that the translocation in the father was a de novo event.

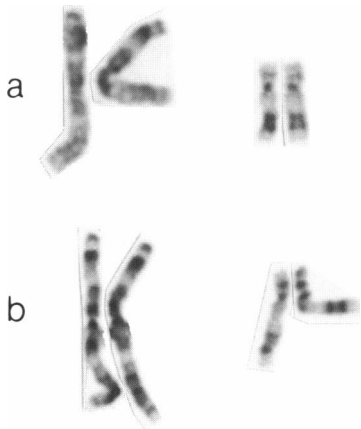

Figure 3 G-banded partial karyotypes of chromosomes 3 and 17 from MDS-18 (*a*) and her mother (*b*). The derivative (17) (p+) is shown on the right of each chromosome 17 pair.

Discussion

The present study demonstrates that nonisotopic in situ hybridization with unique probes is a rapid and sensitive tool for the identification of submicroscopic deletions and cryptic translocations in Miller-Dieker syndrome. The high (75%–95%) efficiency of positive fluorescence signals produced by using two cosmid

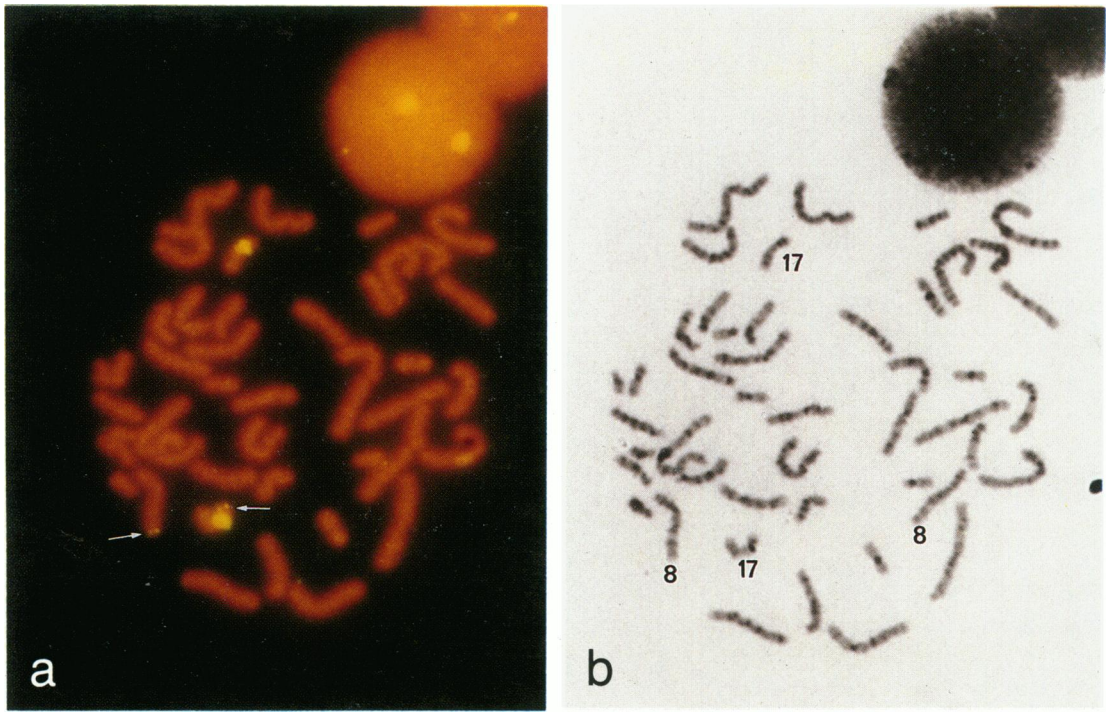

Figure 4 Sequential G-banding of father of MDS-6, demonstrating 8q;17p translocation. FISH (*left*) showed only one cosmid signal on 17p, but a second signal was seen on the distal long arm of a C-group chromosome (*arrow*). G-banding (*right*) showed the signal to be on 8q.

probes enables accurate identification of the chromosomal abnormality after the analysis of 20 cells or fewer. Cohybridization with the chromosome 17 alpha-satellite probe allowed rapid, unambiguous identification of the two chromosome 17 homologues, without banding. Sequential G-banding allowed accurate identification of the translocation chromosomes in two cases. This complete analysis can be done in approximately 2 d from the time slides are made and requires less cytogenetic expertise than does high-resolution chromosome analysis. The only special requirement is the use of a high-quality fluorescence microscope, but nonfluorescence detection methods are also feasible (Zhang et al. 1990).

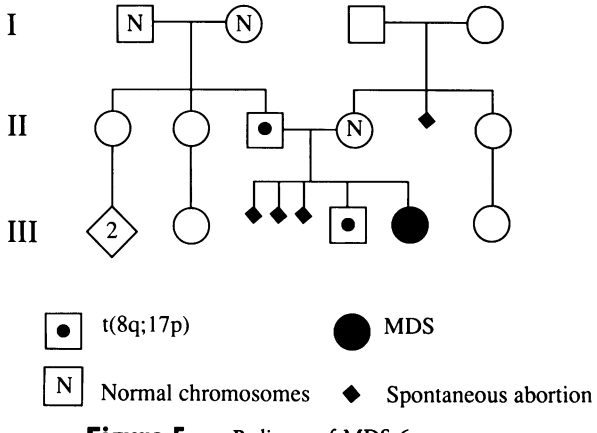

Figure 5 Pedigree of MDS-6

MDS was originally thought to be an autosomal recessive disorder, because of occasional familial recurrence. However, cytogenetically visible or molecular deletions of band 17p13.3 are now known to account for the great majority of cases (Dobyns et al. 1991). High-resolution cytogenetic methods, which are labor intensive and technically difficult, are required for detection of these small deletions. Even with high-quality studies, deletions are only visible in approximately half of cases (Dobyns et al. 1991). A higher frequency of deletions can be detected by RFLP analysis using a panel of five polymorphic markers in this region. One of the five markers, YNZ22, has been converted to a PCR-based typing system which provides a simple, rapid method to detect deletions in some patients (Batanian et al. 1990). However, use of

all five markers by PCR and Southern blotting detects deletions in only about 70% of cases, the remainder being uninformative (Dobyns et al. 1991). In situ hybridization is not dependent on informative polymorphisms, and it is simpler and easier to perform in a clinical cytogenetics laboratory.

Balanced translocations—including translocations between 17p and 7p, 8p, 12q, and 15q—have previously been noted in families with multiple affected MDS children (Dobyns et al. 1984). The present report adds two additional translocation cases, involving 3q and 8q. In the case of the half-cryptic translocation (MDS-18), carrier detection and prenatal diagnosis could have been performed by a combination of (*a*) cytogenetic studies to identify the der(17) and (*b*) RFLP studies to distinguish the balanced from the unbalanced translocation state. In situ hybridization can be used to directly detect the balanced or the unbalanced form of the 3;17 translocation.

For the full-cryptic translocation (MDS-6), the family was initially counseled, incorrectly, that a "normal" karyotype and molecular deletion indicated a de novo deletion with negligible recurrence risk for the couple and other relatives. In situ hybridization studies showed a paternal 8;17 translocation, also inherited by the patient's phenotypically normal brother. In retrospect, this finding probably explains the couple's history of three miscarriages (fig. 5), which may have represented either of the possible unbalanced chromosomal configurations. The father and normal brother have relatively high risk for other MDS children or for unbalanced states leading to miscarriage, but prenatal diagnosis by in situ hybridization is now available. The dramatic change in recurrence risk—and, therefore, in counseling—for this family provides a strong argument for the necessity of performing in situ hybridization studies on the parents of all MDS patients with molecular deletions and "apparently" normal karyotypes.

The greater sensitivity and ease of in situ hybridization compared with high-resolution cytogenetic studies suggests a new approach to laboratory diagnosis of MDS. Routine chromosome studies can first be performed to detect large visible deletions and translocations. If results are positive, parents should also be studied by routine cytogenetic methods to rule out balanced translocations. If the patient has a normal karyotype by routine analysis, in situ hybridization studies can be performed to identify submicroscopic deletions. If a submicroscopic deletion is found by in situ hybridization, parental studies to determine whether the deletion is a de novo event or the result of a cryptic translocation can be performed.

This same strategy, using in situ hybridization with cosmid or other unique probes mapping within the critical deletion interval, could be applied to other microdeletion syndromes, including Prader-Willi and Angelman syndromes (del 15q11–13), DiGeorge syndrome (del 22q11), retinoblastoma (del 13q14), and aniridia/Wilms tumor (del 11p13). Given the technical difficulty and relative inefficiency of high-resolution cytogenetic studies, in situ hybridization approaches may in fact replace high-resolution analysis when a specific microdeletion syndrome is being investigated.

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