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Detection of Chromosomal Aberrations by a Whole-Genome Microsatellite Screen

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Summary

Chromosomal aberrations are a common cause of multiple anomaly syndromes that include developmental and growth retardation. Current microscopic techniques are useful for the detection of such aberrations but have a limit of resolution that is above the threshold for phenotypic effect. We hypothesized that a genomewide microsatellite screen could detect chromosomal aberrations that were not detected by standard cytogenetic techniques in a portion of these individuals. To test this hypothesis, we performed a genomewide microsatellite screen of patients, by use of a currently available genetic-marker panel that was originally designed for meiotic mapping of Mendelian traits. We genotyped ~400 markers on 17 pairs of parents and their children who had normal karyotypes. By using this approach, we detected and confirmed two cases of segmental aneusomy among 11 children with multiple congenital anomalies. These data demonstrate that a genomewide microsatellite scan can be used to detect chromosomal aberrations that are not detected by microscopic techniques.

Introduction

The development and refinement of cytogenetics have led to the characterization of an array of malformation syndromes caused by chromosomal aberrations (Schinzel 1984; Borgaonkar 1994; Verma and Babu 1995). Progressive improvements in cytogenetic banding techniques have allowed for determination of segmental aneusomy, for chromosomal regions of as little as 2–5 Mb of duplication or deletion, under ideal conditions (Ledbetter and Ballabio 1995). However, there exist no data with which to assess the resolution of standard banding techniques performed routinely in a clinical cytogenetics laboratory. The results of studies of contiguous-gene syndromes have demonstrated that duplications or deletions of chromosomal segments smaller than 2–5 Mb can cause multiple anomaly syndromes (Ledbetter and Ballabio 1995; Mazzarella and Schlessinger 1998). Therefore, the lower limit of cytogenetic resolution with the use of G-banding is above the threshold for phenotypic effects.

Polymorphic markers can be used for the detection of segmental aneusomy—specifically, for duplications and deletions (Wilkie 1993). Such an approach contributed to the isolation of the 1.5-Mb duplication in Charcot-Marie-Tooth disease type 1A (Lupski et al. 1991). This approach has also been used for the detection of terminal chromosomal aberrations, by use of VNTR, RFLP (Flint et al. 1995), and microsatellite (simple-tandem-repeat polymorphism [STRP]) markers (Biesecker et al. 1995; Slavotinek et al. 1999). This approach takes advantage of the polymorphic alleles of meiotic mapping markers, to search for non-Mendelian allele-inheritance patterns that are compatible with aneuploidy or segmental aneusomy. These are most readily recognized when a marker is fully informative and when an offspring has either hemizygosity (e.g., shows only one of the four alleles) or trisomy (e.g., shows three alleles). In this way, meiotic markers can serve as probes for duplications and deletions. Previous study groups have focused on terminal chromosomal aberrations, since they are a common subtype of chromosomal rearrangement and since a panel of informative subtelomeric markers can be combined into a robust screen for such aberrations (Flint et al. 1995; Slavotinek et al. 1999). In contrast, the design of

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a marker panel for interstitial aberrations is more challenging. Since each mating is fully informative for only a fraction of markers and since the sensitivity of the whole-genome screen is dependent on the density of informative markers, the panel requires a large number of informative and evenly spaced probes for a robust assay. In this pilot study, we used an existing meiotic mapping panel for the purpose of screening for duplications and deletions, to assess both the practicality and the usefulness of such an approach.

Material and Methods

Patients

Seventeen families from two diagnostic categories were analyzed in this study. Eleven of the children had multiple congenital anomalies and were selected from a pool of 120 such children from a larger study of a subtelomeric screening protocol. The 11 children were selected because the available quantity of DNA was large and because the results of a subtelomeric screen for deletions and duplications, done by use of microsatellite markers, were normal (data not shown). The clinical criteria for this group included: (1) at least three minor anomalies or a major and a minor congenital anomaly, (2) developmental or growth retardation, (3) no affected first-degree relatives, (4) a normal GTG-banded karyotype, (5) no syndromic diagnosis, and (6) no known consanguinity. Six children with VACTERL association were selected, by similar means, from a pool of 80 children. The diagnostic criteria for this group were (1) the presence of three or more of the six anomalies that comprise this association, (2) a normal GTG-banded karyotype, (3) no affected first-degree relatives, and (4) no known consanguinity. The study was reviewed and approved by the National Institutes of Health National Cancer Institute institutional review board. G-banding karyotype analysis was performed by use of standard techniques.

Genotyping

Semiautomated genotyping was performed with the use of a standard whole-genome marker panel (Center for Medical Genetics, Marshfield Medical Research Foundation). The markers in this panel are spaced for optimal genetic distribution, not for physical distribution. To estimate the physical spacing of the marker set, we searched the integrated mapping resource (The Genetic Epidemiology Research Group, Department of Human Genetics, University of Southampton) for markers that were present in the genetic mapping panel (Marshfield screening set, version 7.0). We then tallied all of the intervals of adjacent markers and plotted those intervals, whether physical (in Mb, $n = 305$) or genetic (in cM, $n = 348$), to estimate the difference in genetic and physical spacing of the panel (fig. 1). Manual genotyping was done by incorporation of $\alpha[^{32}P]$-dCTP, as described elsewhere (Biesecker et al. 1995).

For each marker, genotypes both of patients and of their parents were compared to detect a missing allele (deletion or uniparental isodisomy [UPID]), the presence of a third allele or unequal intensity of two alleles (duplication), or the presence of two alleles from one parent and no alleles from the other parent (uniparental heterodisomy [UPHD]). Deletions and UPID were distinguished by the presence or absence of signals from FISH probes on homologous chromosomes, indicating UPID or a deletion, respectively.

Screening of Libraries to Isolate Clones for FISH Probes

For the chromosome 9 experiments, filters from a P1-derived-artificial-chromosome (PAC) library were screened by hybridization. Seven filters containing human PAC clones (Genome Systems) were screened with the use of multiple STRP probes. Each probe was synthesized from a PCR fragment that had been amplified by use of the appropriate STRP primers. Fifty nanograms of a PCR fragment and 5 μl of a 10-μM mixture of forward and reverse primers were combined. The volume was increased to 33 μl with water and was then incubated at 95°C for 5 min. The solution was placed on ice, and 5 μl 10 × KGB buffer (1 × 100 mM potas-

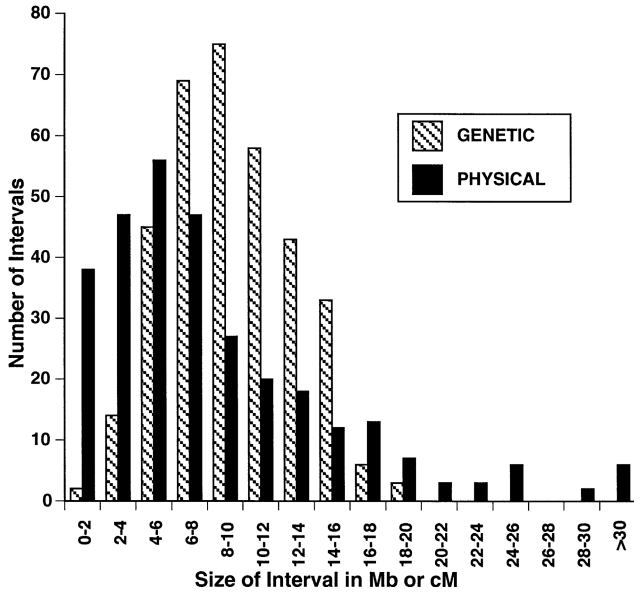

Figure 1 Distribution of markers used to screen subjects. The two data sets represent the spacing of the Marshfield panel, version 7.0, in either genetic distance (*diagonally hatched bars*) or physical distance (*blackened bars*). The genetic spacing of the panel is 10 ± 3.5 cM, whereas the physical spacing is estimated to be 8.4 ± 7 Mb.

sium glutamate, 25 mM Tris-acetate, pH 7.6, 10 mM magnesium acetate, and 50 μg BSA/ml); 5 μl 2.5-mM dATP, dGTP, and dTTP mix; 5 μl α[^{32}P]-dCTP (3,000 Ci/mmol); and 2 μl Klenow polymerase were added. The reaction was incubated at 37°C for 30–60 min. Unincorporated nucleotides were removed by use of the Qiaquick nucleotide removal kit (Qiagen). Filters were prehybridized in roller bottles (no more than two per bottle), with use of standard formamide hybridization solution (Sambrook et al. 1989), at 42°C for 2 h. Probes were pooled and were mixed with 10 μg human Cot1 DNA (Life Technologies), 10 μg poly dCA·dGT (Amersham Pharmacia Biotech), and 100 μl 10-mg/ml sheared DNA (Research Genetics). The mixture was denatured at 95°C for 5 min and then was incubated at 42°C for 20 min. The probe mixture was equally divided and was added to the prehybridization mixture in each bottle. Filters were incubated overnight and were washed the following morning, in 0.1 × SSC/0.1% SDS, at room temperature for 15 min. The wash was repeated at 50°C for 20 min. Filters were exposed to film for 2–3 d, were rewashed in 0.1 × SSC/0.1% SDS at 60°C for 20 min, and were exposed to film overnight. The autoradiographs from the longer exposure were used to orient the filters, and the autoradiographs from the shorter exposure were used to select positive clones. Positive clones were verified by means of PCR done with the use of primers for the STRP probes. DNA was extracted by means of standard alkaline lysis techniques. For the chromosome 1 experiments, a P1 library was screened, by means of PCR amplification of D1S1656 on a pooled arrayed library (DuPont-Merck), by use of standard techniques. Chromosome 19 cosmids were obtained from Lawrence Livermore National Laboratory.

FISH

The cosmids, PACs, and P1s were directly labeled with Spectrum Orange (Vysis), by means of nick translation, for use as probes in FISH studies. Probes were mixed with Cot-1 DNA (Boehringer Mannheim) in a hybridization solution of 50% formamide in 2 × SSC and were denatured at 75°C for 10 min. Metaphase preparations from lymphoblastoid cells were dropped on glass slides by means of standard techniques. The preparations were treated with a graded ethanol series (70%, 80%, and 95%) for dehydration and were denatured in 70% formamide in 2 × SSC at 72°C for 3 min. The cosmids and PACs were hybridized to the preparations, and a reference probe for the chromosome of interest was included in the hybridization. After hybridization, the slides were washed in 1 × SSC at 72°C for 5 min, were treated for detection of the digoxigenin with a fluorescently labeled antidigoxigenin antibody (Oncor), and were counterstained with 4′, 6′-diamidine-2′-phenylindole dihydrochloride (Boehringer Mannheim). The metaphases were analyzed with a Zeiss Axiophot microscope equipped with an Applied Imaging Cytovision system for FISH analysis. Metaphase preparations that showed hybridization of the reference probe on both homologues were scored for the presence or absence of the signals. Ten metaphase preparations were scored for each hybridization.

Results

Whole-Genome STRP Scanning

The study was performed on 17 children with multiple congenital anomalies and on their normal parents. Eleven of these children had undiagnosed multiple congenital anomalies, and six had the VACTERL association. Twelve of the families were genotyped by means of the Marshfield marker screening set, version 6.0, which consists of 393 di-, tri-, and tetranucleotide repeat markers, and five families were typed by means of the screening set, version 7.0, which consists of 396 markers. The genotypes of the children were compared with those of their parents, to determine informativeness and to search for families that had evidence of non-Mendelian inheritance suggestive of segmental aneusomy or uniparental disomy (UPD) (Wilkie 1993). This approach generated 20,088 potential genotypes, of which 346 were uninterpretable, for a failure rate of 1.7%. Because the failure of one, two, or three genotypes for a single marker within a nuclear family commonly results in the inability to determine inheritance patterns, failures were counted by family and not by individual. The total number of markers genotyped for all 17 families was 6,696. Of these, 328 had amplification failure of at least one family member's DNA, for a failure rate of 4.9%. All genotypes that failed to generate an interpretable result were repeated manually by use of α[^{32}P]-dCTP labeling, gel electrophoresis, and autoradiography. Fifty-three of the manually repeated genotypes failed to give a usable result, yielding a final failure rate of 0.8%. The interpretable genotypes were reviewed manually for evidence of non-Mendelian inheritance.

In the initial scan, there were 42 markers for which allele inheritance patterns were incompatible with Mendelian inheritance patterns. In 27 of these non-Mendelian results, the child had an allele that was not present in either parent, and these genotypes were repeated manually. Twenty of them were reproducible, and these alterations were judged to be STRP mutations. The frequency of mutations in this sample set (~.3%) is comparable to results described elsewhere (Weber and Wong 1993; Brinkmann et al. 1998). Nonpaternity was excluded in all cases, because of the large number of other markers that were compatible with paternity.

There were 12 markers from the initial scan for which the child was missing an allele. After reamplification with either the same or a different set of primers for each marker, only four remained abnormal. Finally, there was one marker with a genotype that was compatible with trisomy, since the child's sample had three alleles, and there were two markers with genotypes that were compatible with UPHD. In both cases of apparent UPHD (in family 57 with D19S714 and in family 161 with D2S2944, located on chromosome 2), the child was missing a paternal allele. Flanking markers showed the presence of a paternal allele in both cases. Although the possibility of a small region of segmental UPHD cannot be excluded, it is more likely that the non-Mendelian pattern is the result of a mutation in a paternal allele, so that the final repeat length is, coincidentally, the same as that of the mother's other allele.

For all interpretable markers, we calculated the percentage that excluded the presence of a duplication or a deletion. The sensitivity of the markers used in this study was 77% for monosomy (deletion) and 60% for trisomy (duplication.) Among the genotypes ascertained in this study, there were four loci among three families where an abnormal STRP result suggesting a duplication or deletion was detected and confirmed. These results are described in the following sections.

Family 14

Family 14 had three anomalous genotypes, two of which (D4S1644 and D4S1625) suggested a deletion of maternal alleles for adjacent markers on chromosome 4q (fig. 2*A*). Because null alleles can cause false-positive results for a deletion, the genotypes were confirmed by manual genotyping performed with either the published primers or with multiple custom primer pairs. An additional 41 markers on chromosome 4q were genotyped to confirm the finding and to define the size of the 4q deletion (data not shown). These markers were selected from the Southampton integrated map database (Collins et al. 1996). Ten of these 41 markers also showed missing maternal alleles. These data suggested that the child had a deletion of 11–15 Mb of chromosome 4q. On the basis of these results, a repeat karyotype at 500–550-band resolution was performed; it showed 46,XY,del(4)(q28q31.3) (fig. 2*B*). When the original karyotype (which was reported as normal) was reviewed, it was apparent that the quality of the study was suboptimal and that the deletion was not detected because of the poor resolution of that study. In addition to the results for chromosome 4, a marker on chromosome 1q (D1S1656) was found to be compatible with a paternal deletion (data not shown). The results of analyses of 16 flanking markers on chromosome 1q demonstrated no other genotypes that were suggestive of a deletion (data not shown). Marker D1S1656 was used to isolate a P1 clone, which was then used for FISH analysis. This probe showed a signal on both copies of chromosome 1 in all metaphase spreads, suggesting that the child and father carry a null allele for this marker or that an allele mutation has occurred in the child (data not shown). However, the presence of a deletion that is too small to be detected by FISH cannot be excluded.

The affected child in family 14 is 9 years old and has multiple minor anomalies with developmental and growth retardation and microcephaly. On physical examination, he had a flat mid-face, retrognathia, simple and asymmetrically placed ears, down-slanting palpebral fissures with epicanthal folds, intermittent nystagmus, and 5th-finger clinodactyly. These features are nonspecific but have previously been described in interstitial deletions of chromosome 4q (Lin et al. 1988).

Family 143

Semiautomated genotyping results for marker D19S245 did not detect a paternal allele in this child (fig. 3*A*). Ten additional markers were tested, with three of them confirming the absence of a paternal allele. The size of the monosomic segment was estimated to be 9–11 Mb from the Southampton integrated map in band 19q12 or 19q13.1. Repeat karyotyping with resolution of up to 600 bands showed no clear deletion of material in this region of chromosome 19 (fig 3*B*, inset). FISH analyses done with the use of four probes (23036, 17755, 30016, and 20809), each of which contained one of the four hemizygous STRP markers, showed an absent signal on one chromosome 19 homologue, in metaphase spreads of lymphoblasts from the proband (fig 3*B*). No deletions were detected in the parental metaphases, by use of these four probes (data not shown).

The clinical features of this child included hypotonia, developmental delay, and visual problems, including astigmatism, hyperopia, and esotropia. He had minor dysmorphic features, including dolichocephaly, a high frontal hairline, prominent and low-set ears, a low dermal ridge count, and hypoplastic toenails. He has undergone tracheal reconstruction for subglottic stenosis and strabismus repair.

Family 24

In family 24, marker GATA62F03 (listed as both D9S2169 and D9S935) was reported, from the semiautomated genotyping screen, as a nonamplification in the child and as having bands of unequal intensity in both the child and the father. Reamplification of this marker revealed that the child had three alleles

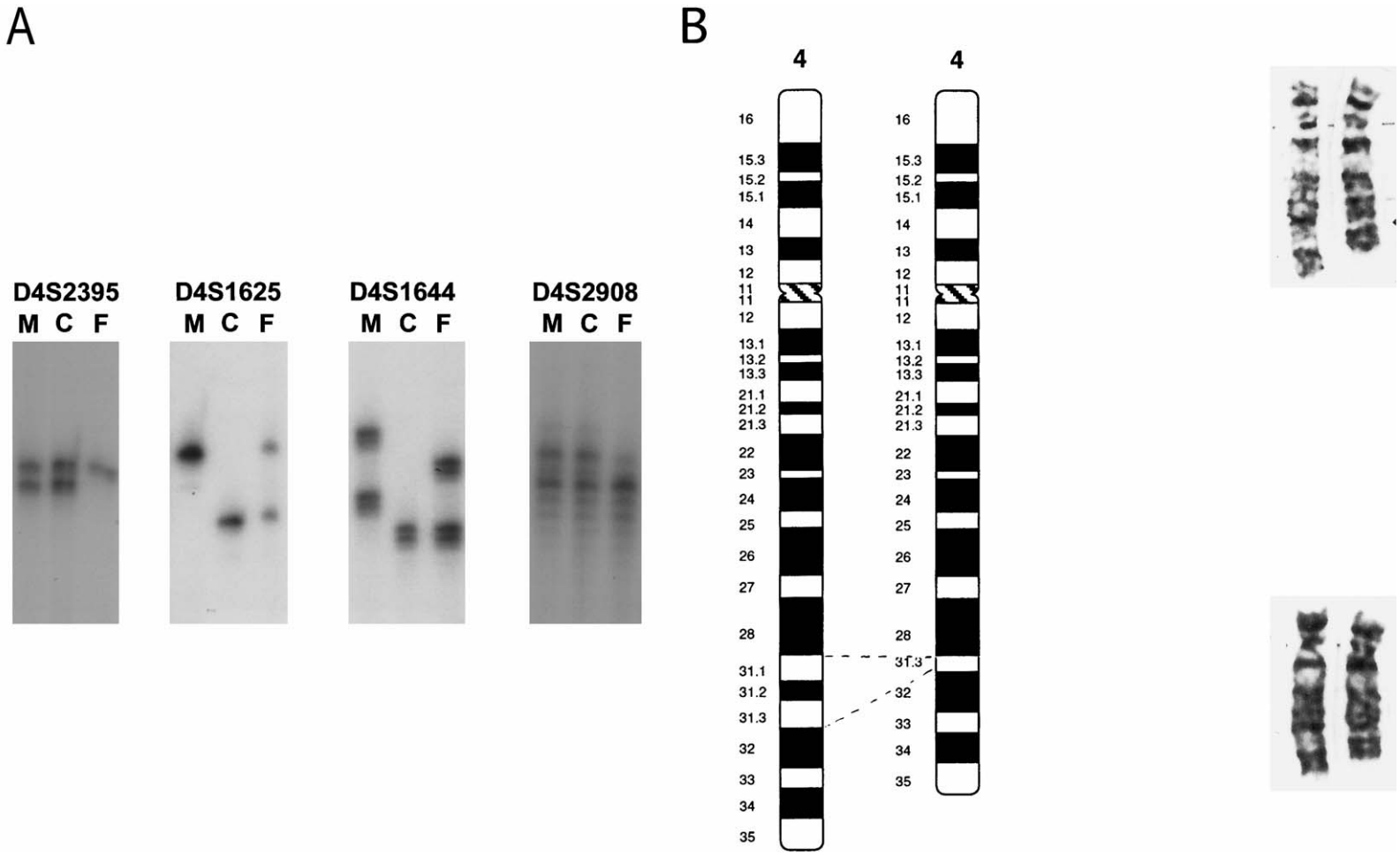

Figure 2 Genotypic and cytogenetic analyses of family 14. *A*, Genotyping results of representative markers from the deleted region on chromosome 4q. D4S1644 and D4S1625 were part of the whole-genome-scan marker set and were repeated by use of radioactive genotyping. Both markers indicate that the child is missing a maternal allele. D4S2395 and D4S2908 are the closest flanking markers that exclude a deletion. M denotes mother; C, child; and F, father. *B*, Partial karyotype of the affected child shows a deletion of 4q28q31.3.

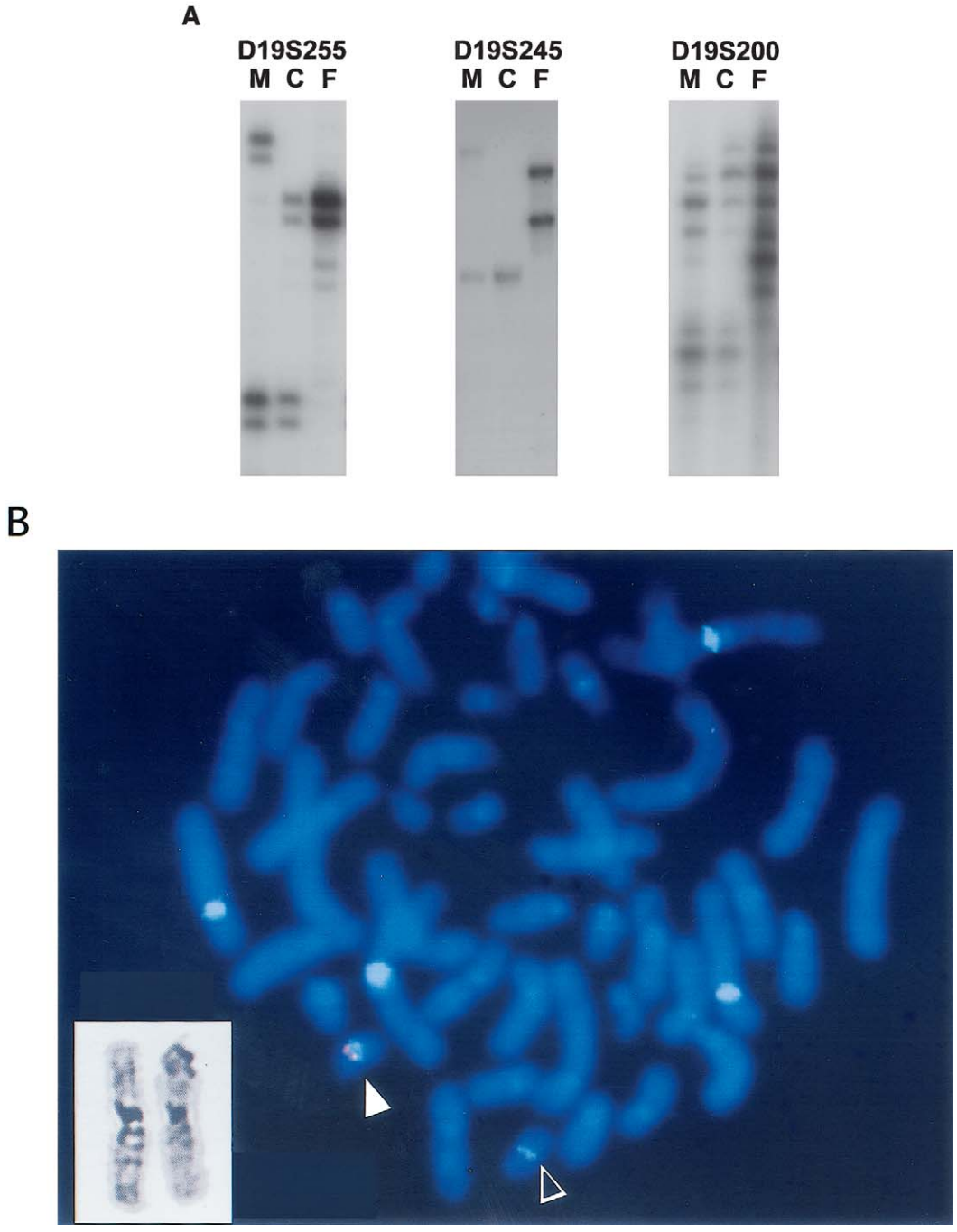

Figure 3 Genotypic and cytogenetic analyses of family 143. *A*, Genotyping results of representative markers from the deleted region on chromosome 19q. D19S245 was part of the whole-genome-scan marker set and was repeated manually. The results show that the child is missing a paternal allele. D19S255 and D19S200 are flanking markers that demonstrate biparental inheritance of alleles. M denotes mother; C, child; and F, father. *B*, FISH analysis performed with the use of PAC probe 23036 that contained D19S414, which was hemizygous by STRP analysis. This probe shows a signal from only one chromosome 19 homologue in the affected child. The inset of a partial-karyotype G-band analysis of lymphocytes from the child shows no visible deletion of chromosome 19q at the 600-band stage. The normal G-band pattern and p:q arm ratio are preserved. The p arm on lower-right chromosome 19 appears to be dark because it was overlapped in the metaphase.

of equal intensity and that the father's 291-bp allele was darker than the 283-bp allele, suggesting that he may be trisomic as well (fig. 4). Detection of trisomy by the observation of unequal intensity of STRP alleles has been described elsewhere (Stone et al. 1996). Twenty-three additional markers from this region were amplified. None had genotypes that confirmed the duplication in the child or that had evidence of unequal band intensity. The most likely explanation for these results is that one of the father's chromosome 9 homologues has both a 291- and a 283-bp allele for GATA62F03 and that his other homologue contains one 291-bp allele. The child then inherited, from his father, a chromosome containing the duplicated marker and, from his mother, a normal chromosome containing a 287-bp allele.

A PAC clone that was isolated (using GATA62F03 as a probe) and then used for FISH analysis of the child showed two signals on 9pter in all metaphases examined, indicating that the duplication must be small. Fiber FISH was performed and did not show evidence of a duplication. Because the duplication is likely to be present in both the father and the child, it is unlikely that this finding is a cause of the anomalies in the child. However, we cannot exclude the possibility of an imprinting effect, although current data show no evidence of imprinting on chromosome 9 (Ledbetter and Engel 1995).

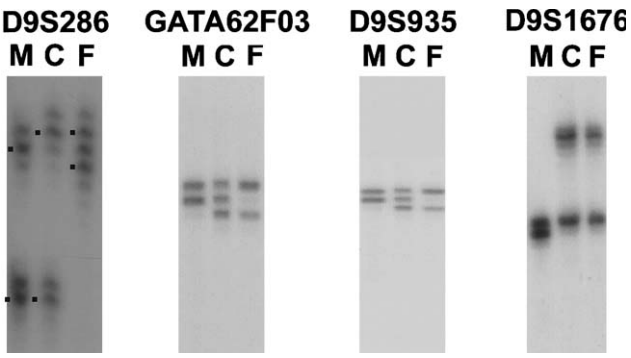

Figure 4 Genotypic analyses of family 24. GATA62F03 (D9S2169 or D9S935) was part of the whole-genome-scan marker set and was repeated radioactively. D9S935 uses a different primer pair to amplify the same repeat as GATA62F03 (D9S2169). Both clearly show both the presence of three alleles in the child's sample and a consistent intensity difference of the father's upper and lower alleles. D9S286 and D9S1676 are nearby flanking markers that exclude trisomy and that do not show an intensity difference between the father's alleles. M denotes mother; C, child; and F, father.

Discussion

This study was designed to test the feasibility of using a whole-genome STRP-scanning approach for the detection of segmental aneusomy. It was not designed to compare the positive or negative predictive power of this approach compared with that of current technology, primarily GTG-banded karyotyping. Instead, we sought to learn how the STRP approach might be implemented, what technical problems might be encountered, and how positive screening results could be evaluated. This study did not include a review of either the previous karyotypes of the patients or the repeat standard GTG-banded karyotypes. This was a deliberate strategy, used to allow inclusion of patients who have chromosomal aberrations that were unknown to us but that may be detectable by a STRP whole-genome screening technique. Other studies have used genetic markers to search for terminal rearrangements, with yields of 6%–18% when adjusted for the informativeness of the markers (Flint et al. 1995; Slavotinek et al. 1999). This pilot study explored the feasibility of generalization of the STRP approach from a terminal-rearrangement screen to a whole-genome screen.

The results of this study demonstrate three important considerations that can be applied to any whole-genome segmental aneusomy molecular-screening technique. First, the technique can detect chromosomal aberrations that are not detected by GTG banding, since they are of inadequate band resolution. One of the subjects (from family 14) in this study had results of a chromosomal analysis that, on retrospective review, were found to be significantly substandard. This chromosome analysis, although adequate to exclude aneuploidy, was inadequate for the detection of segmental aneusomy. However, that such an individual was found allows for a proof of principle that microsatellite screening can detect such aberrations. This result is not surprising, but it afforded an opportunity to evaluate the approach and to demonstrate how STRP screening can detect such abnormalities and how they would subsequently be confirmed by means of high-quality GTG-banded cytogenetics directed at the region determined by abnormal STRP results.

Second, the microsatellite-screening technique will generate false-positive results because of genomic polymorphisms that are not related to the abnormality in the patient (e.g., as in family 24 and family 14, for the chromosome 1q marker). This is an important consideration, since the generation of such results necessitates follow-up studies to either confirm or refute the findings. Such follow-up studies would need to be individualized for each patient and would be challenging and expensive to implement clinically with current technology.

Third, the technique has the potential to diagnose aberrations that are difficult or impossible to detect with high-quality GTG-banded cytogenetic analysis (e.g., as in family 143). In contrast to the findings for family 14, the original karyotype of the child in family 143 was of good quality, and a repeat karyotype, at 400- and 600-

band resolution, was normal—even with the knowledge of the chromosome 19q molecular data. The aberration was confirmed only by FISH studies, by use of cosmid probes that included the aberrant STRP markers. The ability to detect such alterations would be very useful both for clinical care and for the isolation of genes that are important for normal development. All three of these results were obtained in patients who had undiagnosed multiple congenital anomalies; none were found among the six patients with VACTERL association. In addition, both of the aberrations that were confirmed were deletions, and no duplications or disorders of parental origin (UPD) were detected. This may be attributable to the fact that genetic-marker analysis for segmental aneusomy has a higher power to detect deletions than duplications (Wilkie 1993), although the sample size of this study was too small to make conclusions about the relative numbers of duplications, deletions, or UPD.

The alternatives to genotyping, for whole-genome screening for chromosomal aberrations, include comparative genomic hybridization (CGH) and multicolor FISH. CGH has been attempted, with some success, for the detection of terminal rearrangements, although the sensitivity and applicability of this technique are not known (Ghaffari et al. 1998). The usefulness of CGH for the detection of unbalanced interstitial structural abnormalities is unknown. The recent development of multicolor FISH techniques shows excellent promise for clinical cytogenetics (Schröck et al. 1996; Speicher et al. 1996; Uhrig et al. 1999). However, the sensitivity of 24-color FISH done, by use of whole-chromosome paints, for the detection of intrachromosomal rearrangements is inadequate. These considerations suggest that genotyping may be more sensitive for the detection of interstitial aberrations, compared with either CGH or 24-color FISH. Currently, the cost of a whole-genome scan is substantial, but it is likely that progress in automation (Wang et al. 1998) will make this technique more affordable. In any case, it is difficult to imagine that any single technique will offer a high degree of sensitivity for all types of structural or numerical chromosome abnormalities. The ongoing development of a variety of techniques for the detection of chromosomal aberrations has created a need for prospective studies to determine which types of tests are most informative and efficient in clinical applications.

In addition to its potential clinical usefulness, the whole-genome microsatellite technique would allow for the determination of the true rate of small cytogenetic aberrations and for the delineation of additional contiguous gene syndromes. The sensitivity of GTG-banded cytogenetics is dependent on the generation of an altered banding pattern by the aberration. Because the density of GTG bands is not uniform throughout the genome, areas with less-distinct banding patterns are more difficult to analyze (e.g., distal chromosome 1p and chromosomes 19 and 22 [Schinzel 1981]). This suggests that current knowledge of the size range and location of clinically significant cytogenetic aberrations is biased by use of the GTG-banding detection technique. High-density microsatellite analyses would allow for ascertainment of such aberrations, independent of the cytogenetic banding pattern. A future challenge will be to develop panels of markers that are spaced at physical intervals, instead of using the genetically spaced marker panels used in this study. In addition, a fruitful approach may include the application of panels with higher marker density in areas of the genome known to have less-distinct banding patterns. Such studies could be used as a research tool to determine the true frequency and distribution of segmental aneusomy and to search for submicroscopic alterations in syndromes that are hypothesized to be the result of these chromosomal aberrations. The use of thousands of markers in carefully selected patients could be a powerful tool for the molecular characterization of these syndromes.

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Electronic-Database Information

URLs for data in this article are as follows:

Center for Medical Genetics, Marshfield Medical Research Foundation, http://www.marshmed.org/genetics/ (for standard whole-genome marker panel used in genotyping)

The Genetic Epidemiology Research Group, Department of Human Genetics, University of Southampton, http://cedar.genetics.soton.ac.uk/public_html/index.html (for integrated mapping resource)

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