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Molecular refinement of the 1p36 deletion syndrome reveals size diversity and a preponderance of maternally derived deletions

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The deletion of chromosome 1p36 is a newly recognized, relatively common contiguous gene deletion syndrome with a variable phenotype. The clinical features have recently been delineated and molecular analysis indicates that the prevalence of certain phenotypic features appears to correlate with deletion size. Phenotype/genotype comparisons have allowed the assignment of certain clinical features to specific deletion intervals, significantly narrowing the regions within which to search for candidate genes. We have extensively characterized the deletion regions in 30 cases using microsatellite markers and fluorescence *in situ* hybridization analyses. The map order of 28 microsatellite markers spanning the deletion region was obtained by a combination of genotypic analysis and physical mapping. The deletion region was divided into six intervals and breakpoints were found to cluster in mainly two regions. Molecular analysis of the deletions showed that two patients had complex rearrangements; these cases shared their distal and proximal breakpoints in the two common breakpoint regions. Of the *de novo* deletions (n = 28) in which parental samples were available and the analysis was informative (n = 27), there were significantly more maternally derived deletions (n = 21) than paternally derived deletions (n = 6) (χ_1^2 = 8.35, P < 0.0001). Phenotype/genotype correlations and refinements of critical regions in our naturally occurring deletion panel have delineated specific areas in which to focus the search for the causative genes for the features of this syndrome.

INTRODUCTION

Chromosome 1p36 deletion syndrome is a newly delineated, segmental aneusomy condition characterized by a wide range of clinical features, including variable degrees of mental retardation, growth delay, seizures and/or abnormal EEGs, hypotonia, developmental delay, early puberty, orofacial clefting or palate anomalies, characteristic dysmorphic features, hearing deficits and cardiomyopathy. The incidence of deletion of 1p36 is estimated to be ~1 in 10 000 (1), although this deletion may be under-ascertained based on the following reasons: (i) unfamiliarity with the clinical phenotype; and (ii) difficulty in visualizing the light-staining, G-negative band constituting the 1p36 region. Advances in cytogenetic technology, such as fluorescence *in situ* hybridization (FISH), have greatly improved the ability to identify submicroscopic deletions. FISH probes specific to 1p36 have been used in the characterization of previously reported deletion cases (2,3). Additional FISH probes have been recently developed and utilized in confirming 1p36 deletion cases (1).

Several chromosomal deletion syndromes, such as Williams syndrome and Prader–Willi/Angelman syndromes have been shown to have consistently sized deletions that lead to classical presentations (4–6). Unlike these examples, prior molecular and FISH studies on 14 patients with 1p36 deletion syndrome showed that the deletion sizes were variable, as were the clinical presentations (1). Phenotypic variability may be due to differences in the physical extent of each deletion, resulting in the loss of different contiguous, dosage-sensitive genes, genomic imprinting or due to the unmasking of certain recessive alleles. There is speculation in the literature as to the basis of phenotypic variability in growth, physical features, congenital anomalies and seizures among individuals with monosomy 1p36. It has been suggested that phenotypic variability may be due to the parental origin of the deletion and the effects of imprinted genes (7,8). Previous work by our group found no clinical evidence for a parent-of-origin effect in the 1p36 deletion syndrome (1). We propose that haploinsufficiency of contiguous, but functionally unrelated, genes in the deletion region are responsible for these phenotypic features. Therefore, refining the sizes of the deletions in affected individuals, in conjunction with phenotype/genotype correlation, will aid in identifying candidate genes within critical deletion intervals.

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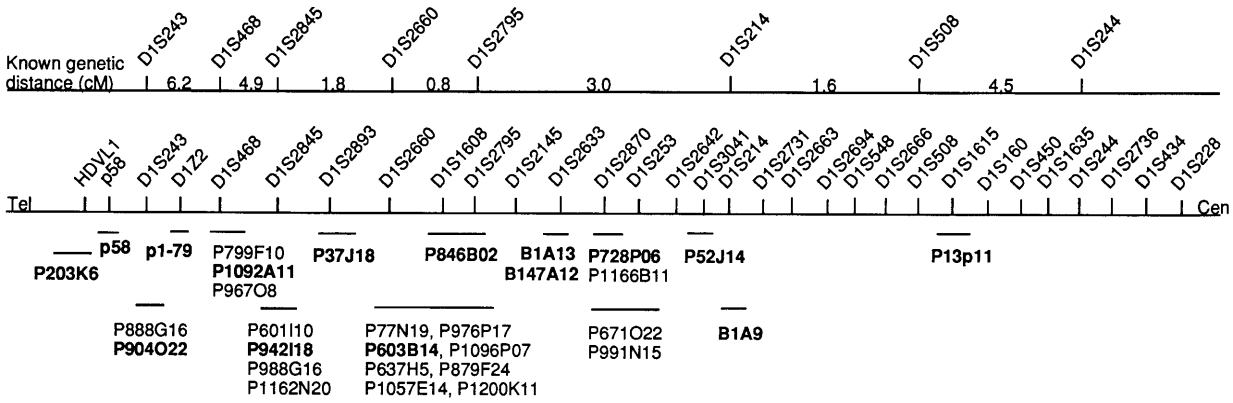

Figure 1. Physical map of 1p36 region. Backbone markers are shown on the top with inter-locus distance in Kosambi cM, based on Vance *et al*. (22). Microsatellite markers are shown on the middle portion of the diagram. Probes are shown below the corresponding locus. B, BAC; P, PAC; p, plasmid. Clones in bold were used as FISH probes. (The diagram is not to scale.)

Although variable, the chromosome 1p36 deletion region may be large (up to ~32 cM) and, as a result of the deletion, multiple loci within the segment become hemizygous. One such previously described gene, the human homolog (*HDVL1*) of the *Drosophila* dishevelled gene (*dsh*), has been mapped within the 1p36 region (9). *HDVL1* was shown to be deleted in a 1p36 deletion patient (10). The *dsh* gene is a developmentally important gene in *Drosophila* and it is functionally conserved in vertebrate embryogenesis (11,12), but the function of *HDVL1* in humans is still unknown. Herein, we describe further investigations of the potential role of *HDVL1* in monosomy 1p36.

The chromosome 1p36 region is believed to contain multiple tumor suppressor genes (13–16). Previous molecular studies identified loss of heterozygosity for 1p36 in some neuroblastomas, as well as other tumor types (17–19). The deleted regions within 1p36 have been characterized in several neuroblastoma cell lines and in one patient with a constitutional deletion of 1p36 who developed a neuroblastoma (20,21). These prior studies indicate that a candidate region for a possible neuroblastoma tumor suppressor gene is within a region of 1p36 which is deleted in some of our patients. Herein, we evaluate the proposed critical regions for neuroblastoma development, relative to the deletion intervals in our panel of patients.

In this study, we investigated 30 patients with monosomy 1p36 through polymorphic microsatellite analysis of 28 markers and FISH analysis with 15 probes. We precisely mapped the distal deletion region, refined the deletion size in each case and determined the parental origin of the deleted chromosome. Finally, we correlated the molecular findings with clinical phenotypes of the patients to begin the delineation of different critical genomic regions associated with particular features of the 1p36 deletion syndrome.

RESULTS

Physical ordering of markers in 1p36

Results of the PCR amplification of P1 artificial chromosomes (PACs) and bacterial artificial chromosomes (BACs) using corresponding markers and all flanking markers are summarized in Figure 1. PCR analysis of PACs 77N19, 976P17, 603B14, 1096P07, 637H5, 879F24, 1057E14 and 1200K11, which all contain D1S2660, also gave positive results for D1S1608 and D1S2795, thus physically linking these markers together. PAC 846B02 contained markers D1S2795 and D1S1608, but not D1S2660, consistent with D1S2660 being either proximal or distal of both D1S1608 and D1S2795. None of these PACs contained D1S2145, therefore, D1S2145 does not lie between D1S1608 and D1S2795. PACs 671O22 and 991N15 were found to be positive for both D1S2870 and D1S253. Combining these results with those of markers previously mapped to 1p36, the likely map order is tel-D1S2660-D1S1608-D1S2795-D1S2145-D1S2663-D1S2870-D1S253-D1S2642-cen.

Three color interphase FISH mapping was performed with five distal 1p36 probes on five normal control individuals (Table 1). Five combinations of markers were used in this analysis (Table 2). Representative results are shown in Figure 2 for four of the five combinations. These results indicate that the most likely map order for the distal 1p36 probes/markers is tel-*HDVL1*-p58-D1S243-D1Z2-D1S468-cen. The physical distance between *HDVL1* and the 1p telomere is not known.

Table 1. Probes used in interphase FISH mapping

Probe number	Probe	Locus
1	P203K6	HDVL1
2	p58	p58 (Cdc2L1)
3	P904O22	D1S243
4	p1–79	D1Z2
5	P1092A11	D1S468

Table 2. Combinations used in interphase FISH mapping

Probe combination	Result	Figure
1, 3, 4	HDVL1-D1S243-D1Z2	Data not shown
1, 4, 5	HDVL1-D1Z2-D1S468	2A
1, 3, 5	HDVL1-D1S243-D1S468	2B
3, 4, 5	D1S243-D1Z2-D1S468	2C
1, 2, 3	HDVL1-p58-D1S243	2D

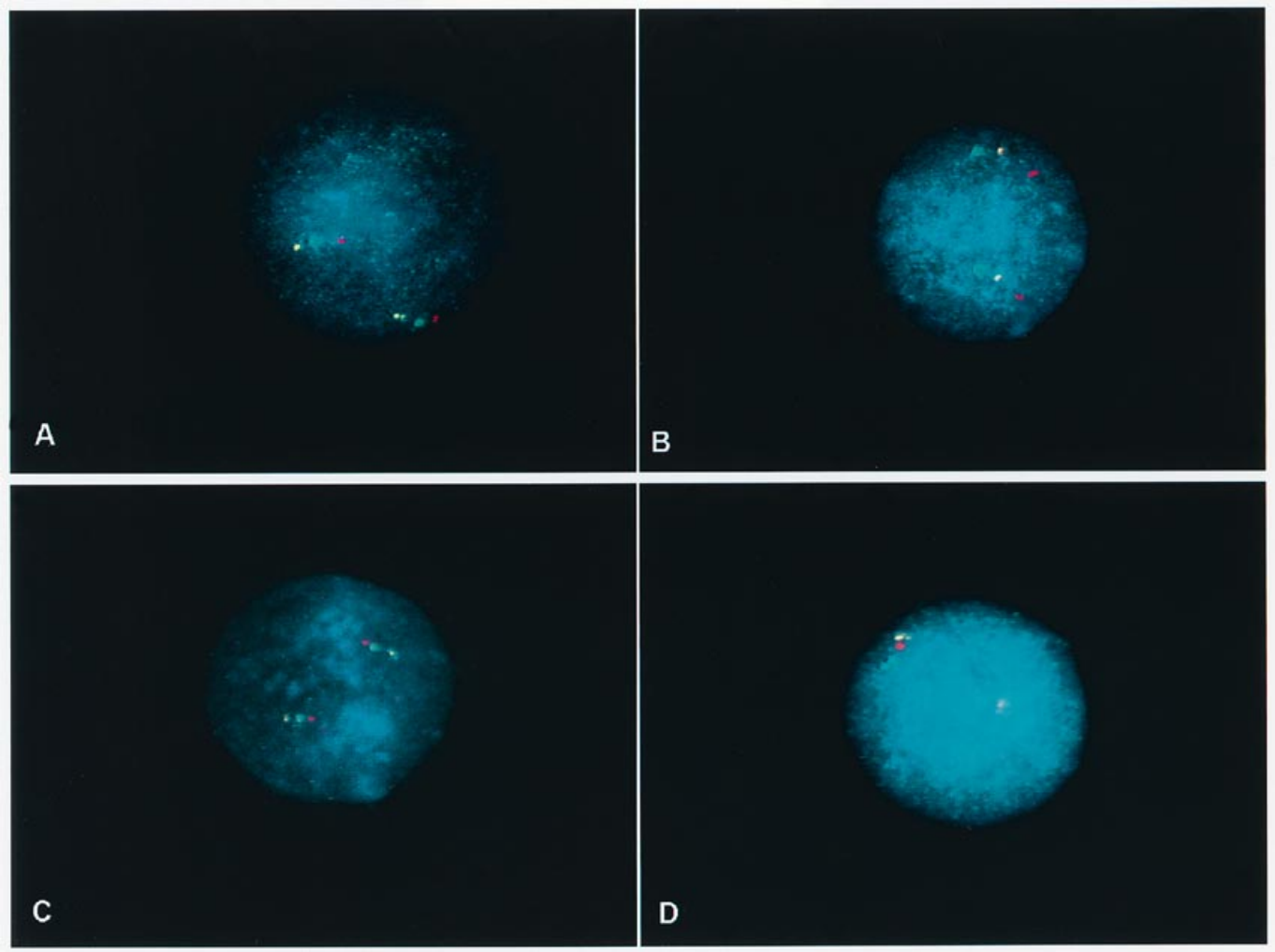

Figure 2. Interphase FISH mapping. Green signals are biotin-labeled probes. Red signals are digoxigenin-labeled probes. Yellow signals are a 50/50 mixture of biotin- and digoxigenin-labeled probes. Interphase mapping result: (**A**) D1Z2 (green), PAC 1092A11 (D1S468, red) and PAC 203K6 (*HDVL1*, yellow); (**B**) PAC 203K6 (green), 1092A11 (red) and PAC 904O22 (D1S243, yellow); (**C**) D1Z2 (green), PAC 904O22 (yellow) and PAC 1092A11 (red); (**D**) PAC 203K6 (yellow), p58 (red) and PAC 904O22 (green).

Molecular delineation of the deleted regions: construction of a panel of naturally occurring deletions

The results combining the FISH and genotypic analyses are summarized in Figure 3. Cases 4, 25, 27 and 29 have the smallest deletions of the 30 patients studied to date and case 18 has the most proximal deletion breakpoint. Cases 1, 3 and 18 showed interstitial deletions, with retention of the most distal available marker, *HDVL1*. Cases 3 and 9 have complex rearrangements, with deletion of distal and proximal markers, but retention of segments around D1S2893. FISH results are in agreement with the genotyping results, with two exceptions. Case 9 was shown to be deleted for locus D1S468 by PCR, but was not deleted when analyzed with the corresponding FISH probe (PAC 1092A11). Similarly, in case 10, D1S2633 was deleted by PCR, but not deleted when analyzed with the FISH probe (BAC 1A13). These results indicate the identification of probes that span the proximal breakpoints in these two cases.

Parental origin of deletions

Parental samples from 29 of 30 patients (all except case 3) were obtained to evaluate the parental origin of each deletion. One patient, 13, has an unbalanced translocation, derived from a paternal balanced translocation (1). The remaining 28 cases were shown to be *de novo*, since parental chromosomes were normal. The parental origin of the deletion could be demonstrated in 27 cases. One case, 25, is currently uninformative with available markers. Of these 27 cases, 21 showed maternal deletions and six showed paternal deletions (Fig. 3). There was a significant difference in the parental origin of the deletions ($\chi_1^2 = 8.35$, $P < 0.0001$), with an excess of maternally derived deletions.

Phenotype/genotype correlations in patients with the 1p36 deletion

Molecular characterization revealed variability in the size of the deletions in our 30 patients (Fig. 3). The majority of patients have

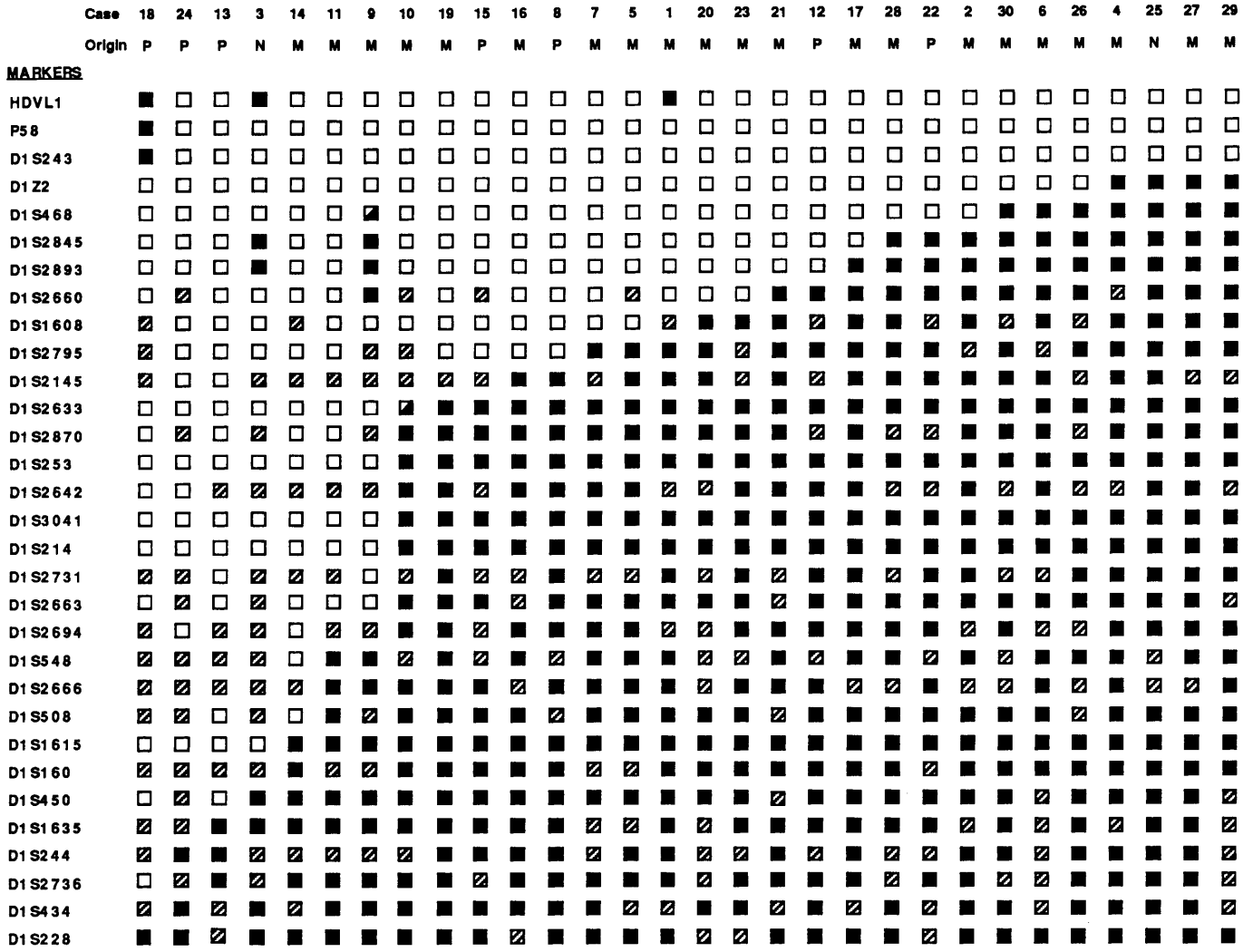

Figure 3. Panel of 30 patients with naturally occurring 1p36 deletions. Below each case number is the parental origin of the deletion. P, paternal; M, maternal; N, unknown. Patients are shown left to right, from the largest to the smallest deletion. Chromosome 1p36 markers are shown distal (top) to proximal (bottom). The FISH probes containing corresponding microsatellite markers are not listed separately on the diagram. Results for FISH and microsatellite analyses are combined. An open square indicates deletion, a solid square indicates non-deletion, a hatched square indicates an uninformative result and a partially shaded square indicates deletion for the microsatellite marker but not deleted for the corresponding FISH probe.

most of the features of the syndrome, regardless of how proximal the deletions extend. Therefore, with few exceptions, most genes contributing to the phenotypic features of the syndrome are in the distal region of the chromosome, specifically distal of marker D1S2870. Additionally, deletions of particular sub-regions of distal 1p36 appear to correlate with the presence of specific features of the syndrome. For example, moderate to severe mental retardation occurs in ~85% of patients with a 1p36 deletion (1). Of the 30 patients described here, it has been observed that those deleted for D1Z2 and more proximal markers have moderate to severe mental retardation, but patients with deletions that encompass D1S243, but not D1Z2, have mild mental retardation and retain complex speech abilities. Therefore, there is likely to be a locus between D1S243 and D1S468 that is critical for cognition and speech.

DISCUSSION

Chromosome 1p36 deletion syndrome is a relatively common segmental aneusomy condition with a specific array of dysmorphic features, congenital anomalies and medical problems. Specific features of the condition may occur when certain sub-regions of 1p36 are deleted, resulting in either hemizygosity for critical dosage-sensitive genes or unmasking of recessive mutations in the non-deleted allele. Physical mapping of particular critical regions within 1p36, along with the development of additional FISH probes for specific sub-regions, will ultimately assist in correlating phenotypic features with the extent of each deletion. This should allow for the refinement of the risks for each patient to develop certain medical problems, such as seizures, hearing loss, growth retardation and mental retardation.

Physical mapping

Prior to our present study, the precise map location of p58 relative to D1Z2 and the most distal microsatellite marker D1S243 was unclear. White *et al*. (20) indicated that D1Z2 was distal to D1S243 and very close to the telomere, based on the molecular characterization of 1p deletions in neuroblastoma tumor cell lines. *HDVL1* was mapped to this region and was placed between markers D1S243 and D1S468 (http://linkage.rockefeller.edu/chr1/data/1p36map/index.shtml). Together, these data indicated a map order of tel-D1Z2-p58-D1S243-*HDVL1*-D1S468-cen. However, since some of our patients retained *HDVL1* but were deleted for D1S243 and D1S468 and other patients were deleted for p58 and D1S243 but not deleted for D1Z2 and D1S468, interphase FISH mapping was performed to clarify the region, which revealed a new map order: tel-*HDVL1*-p58-D1S243-D1Z2-D1S468-cen.

STS content mapping of PACs, in combination with the analysis of the 30 patient samples, has resulted in a new likely map order for 28 STSs and 15 FISH probes in distal 1p36 (Fig. 1). These data modify previously published ordering of markers in this region (20,22; http://linkage.rockefeller.edu/chr1/data/1p36map/index.shtml).

Deletion sizes

Figure 3 displays the 1p36 deletions from 30 patients according to size, decreasing from left to right. Within these, cases 4, 25, 27 and 29 have the smallest deletions, retaining D1Z2, proximally. Although there is no direct measurement of the genetic distance from the telomere to D1Z2, the cytogenetic distance of the smallest deletion size is estimated to be much less than one metaphase band (<5 Mb), based on difficulties in visualizing the deletion using routine cytogenetic methods without the aid of FISH. In contrast, the largest deletions (cases 18, 13 and 24) may encompass a genetic distance of up to 32 cM (23).

The majority of cases appear to be terminal deletions. However, the true 'end' of the chromosome has not been cloned. Therefore, whether the patients have interstitial versus true terminal deletions will need to be determined in future studies. Two cases clearly showed interstitial deletions (cases 1 and 18) and cases 3 and 9 showed complex rearrangements of distal 1p. Our previous analysis of the deletion in patient 4 was reported as a probable interstitial deletion (1), based on the map order known at that time placing D1Z2 distal of D1S243 (20,24,25). However, using our current map order, this patient has one of the smallest, probably terminal, deletions of 1p. Refinement of this deletion panel with additional markers and probes, in conjunction with further phenotype/genotype correlations, will assist in the identification of critical regions in which to search for candidate genes.

Breakpoints

The mechanisms for the multiple breakpoints in this region are unclear. For convenience of the data presentation, we have divided the 1p36 region into six intervals according to the framework of genetic markers based on Jensen *et al*. (26). The six intervals were delineated by the following: I, telomere–D1S243; II, D1S243–D1S468; III, D1S468–D1S2145; IV, D1S2145–D1S508; V, D1S508–D1S244; VI, D1S244–centromere. The markers designated as the junction are assigned from the distal segment (Fig. 4). Thirty-five breakpoints from 28 cases are summarized in Figure 4. Cases 15 and 19 are not included because the boundary markers were uninformative. There appear to be two regions in which the majority of breakpoints in 1p36 occur. One region (II), between markers D1S243 and D1S468, contains 26% of the breakpoints. A second region (III), between D1S468 and D1S2145, contains 43% of the breakpoints. Two cases with complex rearrangements, cases 3 and 9, share both proximal and distal breakpoints in the two common regions. Case 9 is deleted at locus D1S468, but not deleted for PAC 1092A11 (containing D1S468), therefore, this PAC contains one of the breakpoints. Case 10 is deleted at locus D1S2633, but not deleted for BAC 1A13 (containing D1S2633), thus this BAC contains the proximal breakpoint for this case. Identification of PACs spanning additional breakpoints may allow for further understanding of the mechanism(s) resulting in 1p36 deletions.

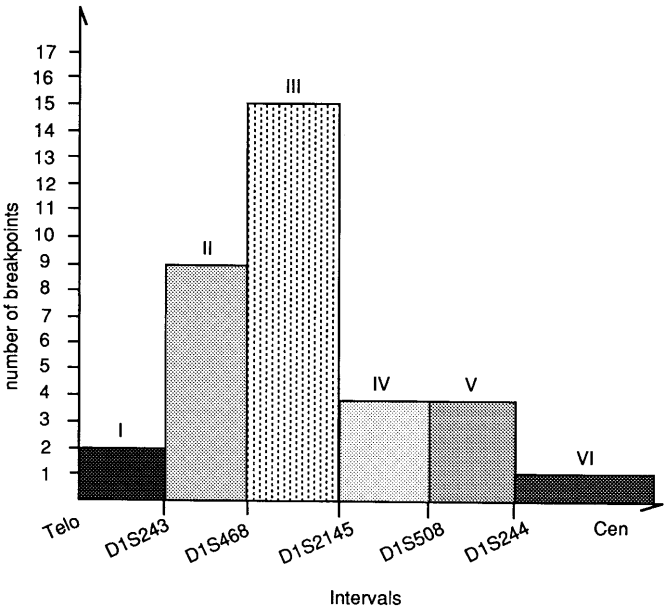

Figure 4. Representation of the common regions of deletion breakpoints within 1p36. The chromosome 1p36 region was divided into six intervals based on the genetic framework of Jensen *et al*. (26). Microsatellite markers are noted on the *x*-axis and the *y*-axis shows the number of breakpoints for each interval. The microsatellite markers shown form the proximal and distal boundaries of each interval.

Parental origin of deletions

The imprinted genes identified to date are not distributed as single genes throughout the genome, but tend to cluster together (27). Well-studied clusters of imprinted genes map to human chromosome 15, where both the Prader–Willi and Angelman syndromes have been localized (28–31), and to human chromosome 11, where Beckwith–Wiedemann syndrome, Wilms tumor and ovarian, breast and lung cancer loci map (32–34). Due to the distribution of imprinted genes over large distances, the mechanism of imprinting may not be location or gene specific (27).

To date, there are no apparent differences in the clinical presentations among our patients with maternally versus paternally derived deletions. However, there are significantly more maternally derived deletions than paternally derived deletions in our study population. Perhaps this reflects an underlying, sex-specific

predisposition to breakage in this region during gametogenesis or, alternatively, could reflect a survival bias due to imprinted genes in this region.

Phenotype/genotype correlations

Since 1987, there have been several cases of presumed pure deletion of 1p36 reported (1,3,8,35–38). In the largest study, we reported the analysis of 13 patients with pure 1p36 deletions and one patient with double segmental imbalance (1). This paper included both clinical and molecular characterization of the deletions. The deletion sizes were found to vary between the patients. Furthermore, it was found that the phenotypic features of the patients varied with the size of the deletions and that patients with larger deletions were more severely affected and exhibited more of the phenotypic features. The present study includes 16 more patients (cases 15–30), additional mapping of the distal 1p region and refinement of the size of each deletion. Patients have been clinically characterized and critical deletion regions that contain genes responsible for several clinical features have been identified. These mapping studies, in conjunction with clinical characterization of the patients, indicate that critical regions associated with certain phenotypic characteristics of the syndrome will emerge. For example, patient 2 has the smallest deletion among the patients with high frequency sensorineural hearing loss, indicating that a gene for high frequency sensorineural hearing loss may reside distal of D1S2845. In another example, with regard to mental retardation, most patients are deleted for all loci distal of D1S468, however, four patients (cases 4, 25, 27 and 29) are only deleted for markers distal of D1Z2. Three of these patients have had cognitive evaluations (the fourth is an infant) and were found to have complex speech abilities and mild mental retardation, while all remaining patients with larger deletions have moderate to severe mental retardation and severely impaired or absent speech. Therefore, the region critical for genes that when hemizygous result in moderate to severe mental retardation is defined distally by D1S243 and proximally by D1S468. Defining critical regions has allowed us to narrow significantly the areas within which to search for candidate genes for various features of the syndrome.

The *Drosophila dsh* gene encodes a cytoplasmic phosphoprotein (39) which regulates cell proliferation by acting as a transducer molecule for developmental processes, including segmentation and neuroblast specification. The human *dsh* homolog (*HDVL1*) maps to chromosome 1p36. *HDVL1* is widely expressed in fetal and adult tissues, including brain, kidney, skeletal muscle and heart. Previous studies showed that the mouse homolog *Dvl1* had abundant expression throughout the neural folds, especially in the spinal cord, in particular on the ventral horns during fetal development (40). Interestingly, one domain of *Dvl1* is similar to a portion of the disc large-1 (dgl) protein, a *Drosophila* tumor suppressor gene (41). Within our 30 patients, cases 1, 3 and 18 are not deleted for the *HDVL1* locus. These three patients do not appear to lack any specific feature found in the remaining patients that could be attributable to the retention of *HDVL1*. Therefore, hemizygosity of *HDVL1* most likely does not contribute to the 1p36 deletion phenotype. Additional evidence that *HDVL1* is unlikely to be involved in the 1p36 deletion syndrome is that mice heterozygous for *Dvl1* have no apparent phenotype, although homozygous knockout mice do exhibit some social interaction problems (42).

Twenty-one years ago, the first deletion of chromosome 1p was detected in a neuroblastoma (13). Since then, cytogenetic analyses of many tumors and cell lines have revealed that these deletions can vary in size, some being very large, with most extending to the 1p telomere. Molecular studies have revealed that chromosome 1p36 is the location of multiple putative tumor suppressor genes, such as *TNFR2* (43), *p73* (44), *Cdc2L1* or *p58*, *Cdc2L2* (45), *DR3* (46), *4-1BB* (47) and *Ox 40* (48). Most of these tumor suppressor genes have been located distal of 1p36.2 by deletion mapping in tumor DNA utilizing Southern blot analysis (49). Recent loss of heterozygosity studies in neuroblastomas defined a critical region bounded distally by D1Z2 and proximally by D1S228 (20). By using microsatellite markers, the critical region for a putative neuroblastoma tumor suppressor gene has been mapped between D1S244 and D1S214 (50–52).

With regard to our patient population, all are deleted for regions distal of but not including D1S228 and in several of our patients the larger deletions could perhaps span a putative neuroblastoma tumor suppressor locus mapping between D1S228 and D1S214 (specifically, cases 3, 9, 11, 13, 14, 18 and 24). However, since none of these patients (ages 2–14 years) has developed neuroblastoma, either the neuroblastoma suppressor locus lies proximal of D1S2736 (the most proximal deleted marker in the patient with the largest deletion) or perhaps factors other than hemizygosity of a locus, such as imprinting, are essential for tumor development.

It has been suggested that a putative neuroblastoma tumor suppressor gene may be subject to genomic imprinting, as 16 of 17 allelic losses in neuroblastoma tumors were found to be of maternal origin (49,53). In addition, a reported case of a 1p36 deletion patient who developed neuroblastoma also had a maternally derived constitutional deletion (21). Of the three cases in our study with the largest deletions in which parental origins could be identified, all had paternally derived deletions and, to date, none of them has developed a neuroblastoma (ages 13, 5 and 2 years for cases 18, 24 and 13, respectively). If a neuroblastoma tumor suppressor gene is located proximally, near D1S160 or D1S228, and is imprinted, then the paternal origin of these large deletions might protect against tumor development. However, if the locus maps more distally, our study group consists of both maternal and paternal deletions in children of all ages (2 months to 18 years) and none of these patients has developed a neuroblastoma, indicating that a role for imprinting in this region, with respect to the development of neuroblastoma, would be unlikely.

MATERIALS AND METHODS

Study population

Thirty individuals with 1p36 deletions were collected for this investigation; data on patients 1–14 have been previously reported (1) and cases 15–30 have been newly ascertained. The overall population of patients identified thus far includes nine males and 21 females and is ethnically diverse, including 15 Caucasian, 11 Latin-American and four African-American individuals. Twenty-eight patients with 1p36 deletions were ascertained by cytogenetics laboratories in Houston and Atlanta and two patients were self-referred to our study. Three cases, 8, 19 and 29, were identified through amniotic fluid chromosome analysis, referred for an elevated maternal serum α-fetoprotein level. In addition, case 29 had abnormal ultrasound findings. Of the 30 cases, one case, 13, was determined to be the result of malsegregation of a

paternal translocation (1) and one case, 3, a paternal sample for chromosome analysis was not available. In the remaining cases (n = 28), the deletions were determined to be *de novo*. Eighteen patients have undergone extensive testing via a General Clinical Research Center (GCRC) protocol to characterize and delineate the clinical features of this syndrome. If not available for the GCRC protocol, clinical records were requested from the referring institution. All clinical data were compiled and examined by one of us (S.K.S.). Blood samples were obtained from all available patients and parents and lymphoblastoid cell lines were established. In case 29, cultured amniotic fluid was used for the molecular investigations. Informed consent, using a Baylor College of Medicine Institutional Review Board approved consent form, was obtained on all study families.

Molecular analysis

Genomic DNA was prepared directly from blood or cultured amniotic fluid samples or established cell lines, using standard protocols. DNA specimens from 30 patients and available parents were analyzed using 28 microsatellite markers. The initial marker order was based on mapping data obtained from the chromosome 1 World Wide Web resource (http://www.med.upenn.edu/~poncol/chr1/resources.htm). These highly polymorphic markers were used in PCR reactions to detect deletions and to determine the parental origin of each deletion. The PCR products were visualized by autoradiography following electrophoresis in 6% denaturing polyacrylamide gels (54).

FISH

Metaphase chromosome preparations of peripheral blood lymphocytes or lymphoblastoid cells from 30 patients were initially analyzed using two FISH probes which map to 1p36.3:p1–79/D1Z2 (ATCC) and p58 (Oncor, Gaithersburg, MD). In cases where the results of polymorphic markers were uninformative, FISH probes containing only the specific STS(s) were used for deletion analysis (Fig. 1). Each probe was labeled with digoxigenin by nick translation. Metaphase chromosomes were prepared using standard protocols and FISH was performed as described elsewhere (55). Additionally, PAC 203K6 (56), containing the human *HDVL1* gene, was also used in FISH analysis of all patients.

Physical mapping of chromosome 1p36 region

PACs (56) were screened by PCR for specific STSs from 1p36.3. Positive PACs were screened with additional STSs from 1p36.3 to establish the STS content of each PAC and to order the PACs relative to one another. This process also refined the relative location of the STSs used.

In order to establish the map order of probes in distal 1p36, interphase FISH mapping was performed simultaneously using three probes, which included combinations of p58, D1Z2, PAC 904O22 (containing D1S243), PAC 1092A11 (containing D1S468) and PAC 203K6 (containing *HDVL1*). For each FISH study, probes were labeled with reporter molecules such that three colors were produced, including one labeled with digoxigenin-dUTP, one labeled with biotin-dUTP and the third labeled with both digoxigenin and biotin. After labeling, 500 ng of each labeled probe were mixed and precipitated in a mixture including 2.5 µg of human Cot-1 DNA, 35 µg of salmon sperm DNA, one-tenth vol of 3 M sodium acetate and 3 vol of 100% ethanol. The probes were diluted in a 50% formamide hybridization solution for a final concentration of 25 ng/µl. Post-washing and detection procedures were the same as used in the standard FISH protocol (55). For each FISH mapping result, at least 500 interphase nuclei (100 on each of five control individuals) were scored in which three signals were linear. The order of the probes was determined when at least 75% of the interphases scored had the same linear order.

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