Molecular characterisation of chromosome 4p deletions resulting in Wolf-Hirschhorn syndrome

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Abstract

We present three patients with Wolf-Hirschhorn syndrome with small cytogenetic deletions of 4p16. One case is a de novo translocation and two cases represent de novo deletions. Using molecular techniques we determined the extent of these deletions and attempted to ascertain parental origin. Case 1 had a deletion of 4p16.3 with a breakpoint proximal to D4S10, case 2 had a larger deletion including D4S62 in 4p16.2, and case 3 had the largest deletion which included D4S240, but not the Raf2 locus in 4p16.1. The parental origin of the deletion in case 3 was paternal; the other two cases were indeterminable. Our results show that these three deletions include the currently proposed Wolf-Hirschhorn syndrome critical region within the most distal 2 Mb of 4p16.3 and offer supportive evidence for continuous terminal deletions.

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by the effects of other deleted genes could contribute to this lack of correlation. The observation of predominantly paternally derived deletions can not be completely explained by imprinting either since the patients with maternally derived de novo deletions or those who have inherited a derivative chromosome 4 from a maternal translocation carrier have classic WHS and are not consistently more severely affected than the patients with paternally derived deletions.^{9 11 14-16} The preponderance of paternal origin of the de novo deletions probably reflects differences in the exposure and susceptibility of male and female germ cells to chromosomal damage which leads to deletion formation.⁷

Many of the previously described WHS deletions were cytogenetically large, including regions of 4p proximal to 4p16.⁴ We present three WHS patients with small cytogenetic deletions. Using molecular techniques we have defined the extent of these deletions, attempted to determine their parental origins, and investigated a correlation between the size of the deletion and the clinical phenotype.

Materials and methods

CASE REPORTS

Case 1. This young girl was the 1900 g product of a 38 week pregnancy complicated by poor weight gain and toxemia (fig 1). A posterior cleft palate was noted at birth and birth weight, length, and head circumference were all below the 3rd centile. The patient began walking at the age of 3 years. At 4 years she experienced a generalised seizure. A karyotype at the age of 6 years showed a deletion of chromosome 4 secondary to a de novo unbalanced translocation. At 17 years her height, weight, and head circumference were below the 5th centile; she was unable to speak and remained incontinent. Her clinical features are summarised in table 1.

Case 2. This male infant was the 1530 g product of a 36 week pregnancy complicated by maternal toxemia and delivery by caesarian section (fig 1). In view of the patient's malformations (table 1) and small size, he was admitted to the neonatal intensive care unit. Blood karyotypes showed a 4p deletion. At 21 months of age he continued to experience failure to thrive, with height, weight, and head circumference still below the 5th centile. Although his vocalisation increased after he received a hearing aid, he is still unable to communicate verbally.

The Wolf-Hirschhorn syndrome (WHS) has an incidence of 1/50 000 live births and is characterised by severe pre- and postnatal growth retardation, severe mental retardation, distinct facial features (fig 1), and a number of other anomalies (table 1).¹² WHS is associated with a deletion of the short arm of chromosome 4 which can occur as a result of a terminal or interstitial deletion, a translocation, or a ring chromosome 4.³ Although previously observed deletions have varied in size, the common region responsible for WHS appears to fall in 4p16.^{13 14}

Previous clinical reports comparing patients' deletions and phenotypes suggest a lack of correlation between the size of the deletion and the clinical severity.¹⁵ A difference in the prevalence of the parental origin of the de novo deleted chromosome was also observed; 17 cases of paternally derived 4p deletions and two maternally derived 4p deletions have been reported.⁶⁻¹³ These observations led to initial speculations of an imprinting role in WHS.¹³ The lack of correlation between deletion size and clinical severity cannot be attributed to imprinting, however, since most patients have deletions from the same parental origin (paternal). Other influences including environmental factors, differences in genetic constitution, or a masking of features

Figure 1 Pictures of three patients with WHS. The picture of case 1 (A) was taken at 13 years of age, case 2 (B) was 13 months old, and case 3 (C) was 11 years old. Case 1 has a deletion of chromosome 4 secondary to a translocation resulting in monosomy 4p and trisomy for another unidentified chromosomal segment. Her facial features presumably reflect the effects of both monosomy and trisomy.

Case 3. This female was the 2100 g product of a caesarian section delivery because of non-progression of labour (fig 1). She was cyanotic at delivery requiring a ventilator for one day and admission to hospital for two to three weeks. Seizures began at the age of 6 months. At 11 years the patient remained well below the 5th centile for height, weight, and head circumference. The patient can walk with assistance and is able to speak a few words. Her clinical features are listed in table 1. Although clinical evaluation suggested WHS, her 4p deletion was not detected by cytogenetic analysis until the age of 10 years.

CYTOGENETIC ANALYSIS

Standard cytogenetic methods were used to produce high resolution G banded cytogenetic preparations from short term lymphocyte cultures.¹⁷ Ethidium bromide was added to the cultures 90 minutes before harvest to enhance chromosome length. Silver staining (AgNOR) was also done on case 1 in order to rule out involvement of an acrocentric short arm in the de novo translocation.¹⁷

PREPARATION OF GENOMIC DNA AND PROBES

Genomic DNA was obtained from EBV transformed lymphoblastoid cell lines from the patients and their parents according to previously described techniques.¹⁸ Interested researchers can obtain these cell lines by contacting Dr Estabrooks or Dr Rao. Probes used in the Southern blot analyses (table 2) and fluorescence in situ hybridisation (FISH) were also prepared according to previously described techniques.¹⁸

SOUTHERN BLOTS

Southern blot analyses using several chromosome 4p16 probes and their respective restriction enzymes (table 2) were performed as described previously.¹⁸ Autoradiograph signals

Table 1 Anomalies observed in the three patients with WHS are listed. The frequency with which the individual clinical features have been observed in previously reported WHS patients are also listed

	Case 1	Case 2	Case 3	Review* (%)
Growth retardation				
Fetal	+	+	+	89
Postnatal	+	+	+	76
Mental retardation	+	+	+	100
Microcephaly	+	+	+	91
Prominent glabella	+	+	+	47
Broad, beak-like nose	+	+	+	64
Hypertelorism	+	+	+	74
Large, simple ears	-(Small)	+	+	69
Micrognathia	-(Prognathism)	+	+	69
Cleft palate	+	+(+CL)	-	57
Renal abn	+	+	UNK	
Strabismus	+	+	-	36
Genital abn	+	+	-	64
CHD (ASD)	-	+	+	55
Downward slanting palpebral fissures	-	+	+	
Bilateral dacryostenosis	-	+	+	
Seizures	+	-	+	47
Contractures	+	-	-	
Ptosis	+	-	-	28
Hearing loss	-	+	-	
Sacral dimple	-	+	-	33
Hypotonia	-	-	+	
Fused teeth	-	-	+	
Nystagmus	-	-	+	
Skeletal abn (scoliosis)	-	-	+	66
Epicanthic folds	-	-	+	26

* Review of 43 WHS patients.¹

Table 2 The results of qualitative and quantitative Southern blot analysis are given. The probe and restriction enzyme used for each locus are listed with each patient's result for the probe and the method by which the result was determined¹⁹

Locus	Probe	Restriction enzyme	Case 1		Case 2		Case 3	
			Status (Method)		Status (Method)		Status (Method)	
D4S141	2R3	HindIII	del	(D)	del	(D)	del	(R)
D4S115	252-3	PstI	del	(D)	del	(D)	del	(D)
D4S95	674	PstI	del	(D)	del	(D)	del	(D)
D4S10	pCos5.52	PstI	del	(D)	del	(D)	del	(R)
D4S62	p8	PstI	not del	(D)	del	(D)	del	(D)
D4S240	pTV73	PstI	not del	(D)	not del	(D)	del	(D)
D4S241	pTV45	PstI	not del	(D)	not del	(D)	not del	(D)
Raf2	Raf2	MspI	not del	(D)	not del	(D)	not del	(D)

del = deleted. (D) = densitometry. (R) = RFLP analysis.

were interpreted using restriction fragment length polymorphisms (RFLPs) or densitometry readings from a Shimadzu scanning densitometer. The chromosome 7 probe KM19 was used as a control probe for densitometry analysis (fig 2).

FLUORESCENCE IN SITU HYBRIDISATION

The acrocentric β satellite probe and chromosome 4 centromeric α satellite probe (D4Z1) labelled with biotin were obtained from Oncor (Gaithersburg, MD). Biotin labelled chromosome paint probes (chromosomes 1, 2, 3, 4, 6, 8, and 12) were acquired from Imagenetics (Naperville, IL). The pC847.351 cosmid probe was provided by Dr Altherr and Dr Wasmuth at UC Irvine. The acrocentric β satellite probe is known to hybridise to β satellite sequences in the cytological satellites and to sequences in the proximal short arm of acrocentric chromosomes; D4Z1 hybridises to alphoid repeats found at the chromosome 4 centromere; and the chromosome paint probes hybridise to unique sequences located throughout the length of the respective chromosome. The pC847.351 probe is a 38 kb cosmid probe which is part of the D4S26 locus and maps 150 kb from the p terminus of chromosome 4.¹⁶

Fluorescence in situ hybridisation was performed according to protocols provided by Oncor (Gaithersburg, MD) and Imagenetics (Naperville, IL). Slides hybridised with the pC847.351 cosmid probe received an additional round of amplification (for a total of two) in order to maximise the signal. Sequential analysis was achieved by initially staining slides for classical cytogenetic analysis, destaining the slides with a 10 minute ethanol exposure followed by two 10 minute methanol exposures, and continuing with the FISH protocol.

Results

CYTOGENETIC ANALYSIS

Case 1 appears to have a deletion of chromosome 4p16.3-pter secondary to a translocation that has remained indecipherable with current cytogenetic staining techniques (fig 3). The AgNOR staining results regarding B group chromosomes were negative, although this technique could not rule out the presence of an inactive NOR. Her karyotype is 46,XX,-4,+der(4)t(4;?) (p16.3;?) de novo.

Case 2 has an apparently terminal deletion of chromosome 4 (fig 3). A trace of G band positive material at the tip of the deleted chromosome and the lack of 4p16.3 suggest a breakpoint at 4p16.2: 46,XY,del(4) (p16.2). Parental karyotypes were normal.

Case 3 also has an apparently terminal deletion of chromosome 4 which appears to include

Figure 2 Examples of qualitative and quantitative Southern blot analysis. (A) A paternal deletion of pCos5.52 (D4S10) in case 3 detected with a restriction fragment length polymorphism using the restriction enzyme PstI. (B) A deletion of p252-3 (D4S115) in case 2 detected with quantitative Southern blot analysis. The DNA is cut with PstI, and the chromosome 7 probe KM19 is used to correct for differences in DNA amounts between lanes. The ratio of the corrected p252-3 signal reading between the mother and the proband is 2.0 to 1, indicating a deletion of p252-3 in the proband.

Figure 3 Examples of the deleted chromosome 4. (A) Chromosome 4 ideogram (850 band level, ISCN 1985²⁰) depicts the cytogenetic breakpoints in the three WHS patients. (B) Partial karyotypes of chromosome 4 from each case. The deleted chromosome 4 is arrowed.

Figure 4 Examples of FISH. (A) A metaphase spread of a normal control showing the hybridisation of the pC847.351 cosmid probe to both chromosome 4 short arms (arrows). (B) A metaphase spread of one of the apparently terminal deletion patients (case 3) showing hybridisation of the pC847.351 cosmid probe to only one chromosome 4 short arm (arrows). A chromosome 4 centromere probe (D4Z1) was used for identification.

Figure 5 Map of WHS deletions. A diagram depicts the three WHS deletions in relation to each other, to the currently proposed WHS critical region,^{18,21} and to the approximate position of the most distal loci.²⁰ Probes pTV45 (D4S241) and pTV73 (D4S40) have been mapped to chromosome 4p16 proximal to D4S62 by somatic cell hybrid analysis (Carlock, unpublished observations). The cosmid probe pC847.351 is designated as (351), maps approximately 150 kb from 4pter, and does not appear to be in the Wolf-Hirschhorn syndrome critical region.^{16,18}

bands 4p16.2 and 4p16.3 (fig 3). Her karyotype designation is 46,XX,del(4) (p16.1). Parental karyotypes were normal.

FISH ANALYSIS

All three WHS cases failed to show hybridisation on one chromosome 4 with the cosmid probe pC847.351, a distal 4p probe (fig 4). Sequential banding (G banding followed by FISH) showed that pC847.351 was consistently missing from the deleted chromosome 4 in 12/12, 14/14, and 11/11 metaphases examined in case 1, case 2, and case 3 respectively. Cosmid probe signals were observed on the normal homologue in each of these metaphases. Metaphases with no cosmid signal were disregarded since they were uninformative. These observations are statistically significant ($p < 0.0001$) using a one tailed t test and indicate that these deletions extend to at least 150 kb from the 4p terminus.

The second chromosome involved in the translocation in case 1 remained unidentified despite FISH with whole chromosome specific paint probes to chromosomes 1, 2, 3, 4, 6, 8, and 12 (data not shown). β satellite and Y long arm probes excluded the involvement of an acrocentric short arm or the Y chromosome long arm (data not shown).

SOUTHERN BLOTTING

The extent of each patient's 4p deletion is illustrated in fig 5. In case 1, the deletion involved approximately 6 Mb, in case 2 approximately 8 Mb, and in case 3 greater than 8 Mb of the distal 4p16 region, based on a current physical map of chromosome 4p16.²¹ Only case 3 provided parental origin information; her deletion was paternal in origin (fig 2).

Discussion

We have defined the molecular extent of chromosome 4p deletions in three WHS patients. There was excellent correlation between the molecularly determined breakpoints and those estimated in the original cytogenetic diagnosis (fig 5). This correlation substantiates the precision of high resolution cytogenetic analysis.

Although the proximal breakpoints of our three patients' deletions differed, they all shared a common deleted region. This region is defined by the smallest deletion, seen in case 1, which falls in 4p16.3 from D4S10 to the 4p terminus (fig 5). This smallest region of overlap among our patients consists of the distal 6 Mb of 4p16.3, based on 4p physical mapping data.²¹

Although these deletions are cytogenetically small, they are not the smallest reported deletions in WHS patients. Recently a WHS patient with a terminal deletion of the distal 2 Mb of chromosome 4p was reported, placing the WHS critical region in the distal third of 4p16.3.²² A previously published report placed the WHS critical region distal to D4S10.⁹ All of our deletions include the D4S10 locus and

the currently purported WHS critical region within the distal 2 Mb of 4p16.3 (fig 5).

We recently reported two families with a satellited chromosome 4 resulting in a deletion detected by probes pC847.351 and B31 with no family history of WHS.¹⁸ Another report describes an affected subject inconsistent with the classic WHS phenotype who had a distal 4p deletion and partial 7q trisomy secondary to an inherited translocation.²² These cases further define the WHS critical region as proximal to the most distal 150 kb.

The deletion of the pC847.351 cosmid probe in the two apparently terminal deletion patients (cases 2 and 3) is consistent with a continuous, terminal deletion since pC847.351 maps only 150 kb from the 4p telomere,¹⁶ but this observation can not disprove an interstitial deletion with a second breakpoint less than 150 kb from the telomere. Investigations with the pC847.351 probe in two additional WHS patients have also shown a deletion of this distal region offering further supportive evidence for terminal deletions.²³ FISH with telomere probes on these two patients confirmed the presence of telomere sequences on the deleted 4p ends (Roulston, personal communication). This suggests that, if terminal, these deletions may have been healed by the addition of telomere sequences on the broken ends, presumably by telomerase.^{24,25}

Our phenotypic data also appear to support previous observations of a lack of correlation between deletion size and clinical severity (table 1). The three patients do, however, manifest various shared and unique clinical features. Some of the unique features observed in case 1 may be attributable to the partial trisomy resulting from her de novo rearrangement. Those features that do not always correlate with overlapping, mutually deleted regions may result from deletions of chromosome 4p gene(s) that are sensitive to influences by environmental, stochastic, or additional genetic factors. The lack of substantial increase in the clinical severity and in the number of specific clinical features observed in patients with larger deletions may also reflect the actual number of genes in the additionally deleted region. Ultimately, identification of the genes normally residing in 4p16 should offer some explanations for these observations.

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