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ARTICLE

On the Nosology and Pathogenesis of Wolf–Hirschhorn Syndrome:
Genotype–Phenotype Correlation Analysis of 80 Patients and Literature Review

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Based on genotype–phenotype correlation analysis of 80 Wolf–Hirschhorn syndrome (WHS) patients, as well as on review of relevant literature, we add further insights to the following aspects of WHS: (1) clinical delineation and phenotypic categories; (2) characterization of the basic genomic defect, mechanisms of origin and familiarity; (3) identification of prognostic factors for mental retardation; (4) chromosome mapping of the distinctive clinical signs, in an effort to identify pathogenic genes. Clinically, we consider that minimal diagnostic criteria for WHS, defining a ''core'' phenotype, are typical facial appearance, mental retardation, growth delay and seizures (or EEG anomalies). Three different categories of the WHS phenotype were defined, generally correlating with the extent of the 4p deletion. The first one comprises a small deletion not exceeding 3.5 Mb, that is usually associated with a mild phenotype, lacking major malformations. This category is likely under-diagnosed. The second and by far the more frequent category is identified by large deletions, averaging between 5 and 18 Mb, and causes the widely recognizable WHS phenotype. The third clinical category results from a very large deletion exceeding 22–25 Mb causing a severe phenotype, that can hardly be defined as typical WHS. Genetically, de novo chromosome abnormalities in WHS include pure deletions but also complex rearrangements, mainly unbalanced translocations. With the exception of t(4p;8p), WHS-associated chromosome abnormalities are neither mediated by segmental duplications, nor associated with a parental inversion polymorphism on 4p16.3. Factors involved in prediction of prognosis include the extent of the deletion, the occurrence of complex chromosome anomalies, and the severity of seizures. We found that the core phenotype maps within the terminal 1.9 Mb region of chromosome 4p. Therefore, WHSCR-2 should be considered the critical region for this condition. We also confirmed that the pathogenesis of WHS is multigenic. Specific and independent chromosome regions were characterized for growth delay and seizures, as well as for the additional clinical signs that characterize this condition. With the exception of parental balanced translocations, familial recurrence is uncommon.

KEY WORDS: Wolf–Hirschhorn syndrome; WHS; 4p deletion

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INTRODUCTION

Wolf–Hirschhorn syndrome (WHS) (OMIM 194190) was first described independently by Wolf et al. [1965] and by Hirschhorn et al. [1965] as a multiple congenital anomalies/mental retardation (MCA/MR) syndrome caused by partial 4p deletion. Clinical signs include a typical facial appearance, resembling the "Greek warrior helmet" profile, mental retardation, severe growth delay, hypotonia, congenital heart malformations, midline defects, such as cleft palate and hypospadias, ocular colobomas, renal abnormalities and seizures [Battaglia et al., 2001]. The early series of WHS patients had large 4p deletions, all associated with a severe phenotype, which until recently was a diagnostic hallmark of this condition. However, an increasing number of very small 4p16.3 deletions, either terminal or interstitial, were identified in the last few years due to the currently available techniques for molecular karyotyping. These small deletions are associated with a mild or an atypical WHS phenotype, making it necessary to revise the diagnostic criteria. There is a consensus that the core WHS phenotype is defined by the association of typical facial appearance, growth delay, mental retardation and seizures (or EEG anomalies). These signs should be considered minimal diagnostic criteria. Their fine mapping within the critically deleted region is relevant for the search of pathogenic genes.

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Although the extent of the 4p deletion varies greatly in individual patients, hemizygosity of the terminal 4p16.3 region is necessary and sufficient to cause the core phenotype. Two distinct regions were independently described as critically deleted in WHS [Wright et al., 1997; Zollino et al., 2003], generating some confusion about what region is to be investigated for the molecular diagnosis of WHS.

With respect to the pathogenesis, it was previously assumed that a single gene could be responsible for the full WHS phenotype, acting as master transcriptional regulator of other genes. Actually, several genotype–phenotype correlation studies point at a multigenic pathogenesis as more likely, and different genes on 4p16.3 have been suggested to cause distinctive clinical manifestations. In particular, haploinsufficiency of *WHSC1* has been related to the facial characteristics, and hemizygosity of *LETM1* to seizures. With respect to growth delay, literature reports are contradictory. A small 4p region for growth retardation was recently identified by two independent observations within the terminal 760 kb [Concolino et al., 2007] and the terminal 1.27–1.46 Mb [South et al., 2007] of chromosome 4p, respectively. However this evidence came from a ring chromosome 4 in both cases, making its interpretation rather dubious [Zollino et al., 2008].

In this article we tried to elucidate the pathogenesis of WHS and to identify specific chromosome regions where critical genes reside by analyzing a total of 80 WHS patients, all carrying a chromosome 4p deletion that encompasses WHSCR-2, and two additional patients with atypical 4p deletion not productive of WHS. We also review the relevant literature reports and discuss the mechanisms of origin of the basic genomic defects and prognostic factors in WHS.

THE WHS CRITICAL REGION

Although they may be of different length, 4p deletions that include the terminal 4p16.3 chromosome region are the basis of WHS. At a molecular level, comparative analysis of either terminal and interstitial deletions allowed the description of the first critical region, WHSCR, limited to a 165 kb interval at about 2 Mb from the telomere, defined by the loci D4S166 and D4S3327 [Wright et al., 1997]. The wide phenotypic effects caused by such small deletion had suggested that only a few pleiotropic genes, perhaps just one, residing in the region, may be responsible for the WHS phenotype, acting as transcriptional regulator(s) of other genes. Two genes, *WHSC1* [Stec et al., 1998] (OMIM 602952), two thirds of which map within the distal half of the WHSCR, and *WHSC2* [Wright et al., 1999] (OMIM 606026), falling entirely within only WHSCR, were described as candidate genes. However, productive mutations have not been detected so far. *LETM1* (leucine zipper/EF-hand-containing transmembrane (OMIM 604407)), which is involved in Ca2 signaling, was recently described as another candidate gene for WHS, in particular for the seizure disorder [Endele et al., 1999].

LETM1 is identified by cosmid 75b9 and PAC clone 184O23, and it maps distally to WHSCR. In spite of some evidence, the role of WHSCR remains questionable. As a matter of fact, most WHS-associated deletions are much larger than WHSCR, usually including all the candidate genes.

A unique patient was reported by Rauch et al. [2001] with a small interstitial deletion restricted to WHSCR and in whom the *LETM1* gene was preserved. As pointed out by the authors, this patient presented with an atypical WHS phenotype that included normal height, subtle WHS facial appearance, and, more importantly, no seizures. Another large interstitial deletion—resulting in an atypical WHS phenotype, with no seizures—established the distal boundary of WHSCR [Somer et al., 1995; Wright et al., 1997]. *LETM1* was preserved also in this patient. All these considerations, together with the detection of a 1.9 Mb deletion productive of the full WHS core phenotype, including seizures, but not including the whole WHSCR, allowed re-definition of the critical region within an interval of 300–600 kb between the loci D4S3327 (at 1.9 Mb from the telomere) and D4S98-D4S168 (at 1.6–1.3 Mb from the telomere) [Zollino et al., 2003]. The newly described region was

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referred to as WHSCR-2. It is contiguous, distally, to the previous one, sharing with it the *WHSC1* gene. It is worth noting that *LETM1* lies within this region. A subsequent report by Rodriguez et al. [2005] confirmed this evidence. Therefore, we consider WHSCR-2 as the critical region for WHS.

CLINICAL CATEGORIES OF WHS

The great variability in the exent of the 4p deletion, as documented by all reported series of patients, makes the resulting phenotype highly variable. Deletions of several megabases in length are associated with a severe phenotype, including typical facial appearance, severe growth delay, severe mental retardations, midline defects, such as cleft palate and hypospadias, congenital heart malformations, renal and skeletal anomalies and seizures.

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The typical phenotypic findings in these cases make the clinical diagnosis of WHS unquestionable. However, an increasing number of very small 4p16.3 deletions were identified in the last few years by molecular karyotyping. Small deletions are usually associated with a mild phenotype, in which clinical signs can only include those defining the core phenotype. This category is likely under-diagnosed.

To facilitate proper genetic diagnosis and counseling, we suggested distinguishing WHS into two different forms, "classical" and "mild," on the basis of clinical presentation, that in turn is related to the extent of the 4p deletion [Zollino et al., 2003]. However, a third clinical category should now be added, including those patients in whom a very large deletion exceeding 22–25 Mb is detected. Thus, from a clinical point of view, three different categories of the WHS phenotype can be defined. The first one, the "mild" form, usually refers to small deletions not exceeding 3.5 Mb. Patients in this group can present with a very mild degree of mental retardation, language can be fluent, independent walking can occur by the age of 2–3 years (Fig. 1). Major malformations are uncommon. The second, and apparently more common category, also referred to as the "classical" form, is associated with a larger deletion, measuring on average 5–18 Mb. Patients in this group present with the typical WHS phenotype, including severe psychomotor delay, delayed or absent speech, late walking (Fig. 2). In addition, major malformations are common. The last, and quantitatively smallest category is identified by large deletions exceeding 22–25 Mb, resulting in a phenotype that one can hardly define as WHS. In addition to severe psychomotor delay, that prevents language development and social skills,

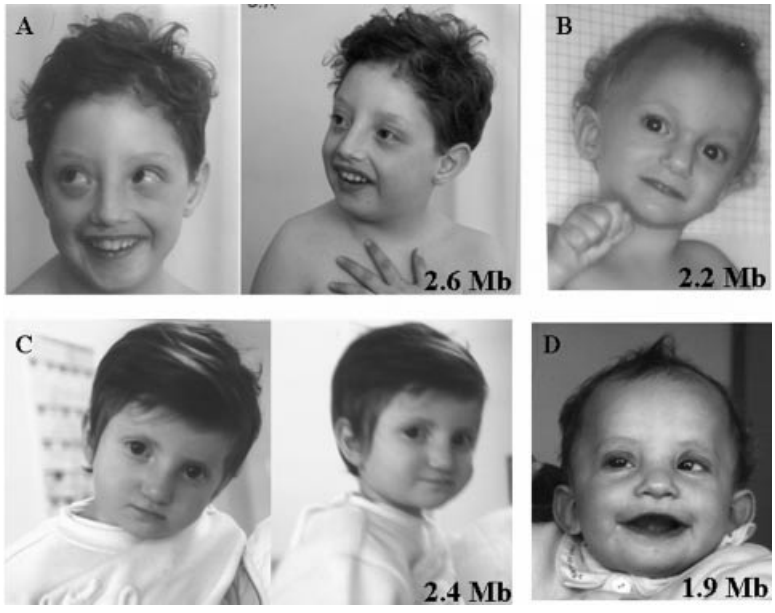

Figure 1. Facial appearance of patients with small 4p deletions not exceeding 3.5 Mb. A good contact with the environment can be noted. **Patients A** (No 13) and **B** (No 15) were reported in Zollino et al. [2000]. Deletion size was slightly modified, on the basis of updated maps [Zollino et al., 2003]. **Patient C** was already reported (Patient 103) in Zollino et al. [2007]. **Patient D** corresponds to patient MG in Zollino et al. [2003].

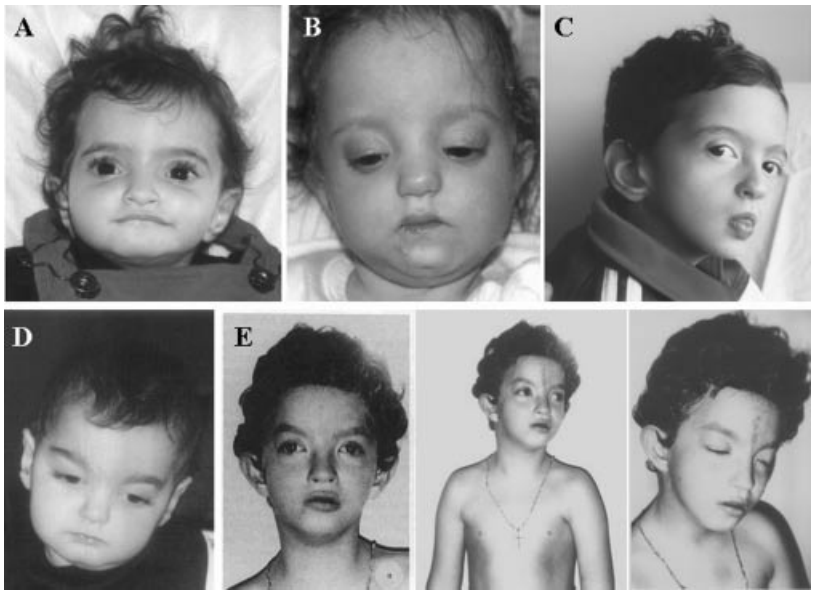

Figure 2. Facial appearance of patients with an average 4p deletion of 5–18 Mb. They were already reported in Zollino et al. [2000] as patient 10 (**A**), patient 7 (**B**), patient 9 (**C**), patient 4 (**D**), and patient 8 (**E**). Pictures of patients C and E refer to an older age.

distinctive of this category are facial anomalies that do not obviously resemble WHS (Fig. 3). Patients usually do not sit without support or walk. Severe scoliosis and psychotic behavior can be additional component manifestations. Clinical signs characterizing these categories are summarized in Tables I–III.

However, it must be stated that phenotypic variability, that is a distinctive feature of WHS, cannot be explained exclusively by variation in the extent of the 4p deletion, as discussed later.

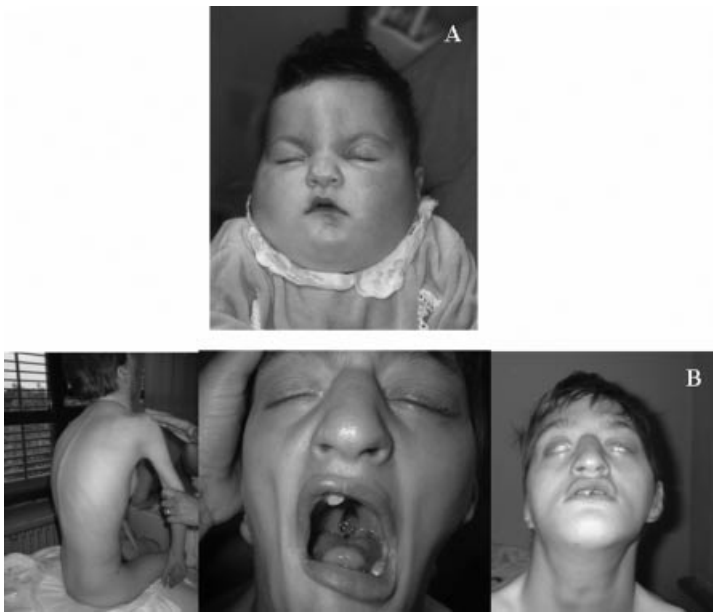

Figure 3. Phenotype of patients with very large 4p deletions, above 22–25 Mb. Facial phenotype appears different from that of typical WHS, and clinical presentation by far more severe. **Patient A** corresponds to patient 5 in Zollino et al. [2000]; **patient B** corresponds to patient 37 in Zollino et al. [2007].

From Tables I–III it can be noted that shared clinical signs include typical facial appearance, growth delay, mental retardation, and seizures (or EEG anomalies). As already mentioned, all these signs are required to make a valid diagnosis of WHS. Atypical deletions not productive of the whole core phenotype represent an important tool in identifying pathogenic genes in WHS. In addition, they suggest that the WHS phenotype is multigenic, always resulting from a chromosome deletion. However, they should not be reported as associated with "atypical WHS."

THE BASIC GENOMIC DEFECT

The WHS-associated rearrangements consist of derivative chromosomes 4 originating from a parental translocation in 10–15% of cases [Dallapiccola et al., 1993; Zollino et al., 2004]. Rearrangements are de novo in the remaining 85–90% of cases. Although de novo rearrangements are largely assumed to be isolated deletions, unbalanced de novo translocations, especially t(4p;8p) translocations, have been detected with unexpected high frequency in WHS patients [Wieczorek et al., 2000b; Tonnies et al., 2001; Giglio et al., 2002; Maas et al., 2007; South et al., 2008a]. Adding the new observations reported here to our previous experience [Zollino et al., 2004, 2007], we were able to establish four different categories of rearrangements: (1) isolated 4p deletions, accounting for about 70% of cases. Pure deletions are usually terminal, but interstitial deletions preserving the 4p subtelomeric region can also occur; (2) unbalanced translocations, accounting for about 22% of cases. They include more frequently t(4p;8p) translocations, but also t(4p;7p), t(4p;11p), t(4p;20q), t(4p;21q), t(4p;12p) and t(4p;Dp/Gp); (3) inverted duplications associated with terminal deletions on the same 4p arm in 6% of cases; (4) derivative chromosome 4, consisting of an unbalanced pericentric inversion that causes a large 4q segment to be duplicated on the deleted 4p, in the remaining 2% of cases (Table IV). Interestingly, a striking correlation was found between type of rearrangement and its

TABLE I. Clinical Signs Associated With Deletions <3.5 Mb (Our Patients: 14; Literature Patients: 36)

	No.	%
Typical facial dysmorphisms	50/50	100
Mild/moderate mental retardation	31/41	76
Severe mental retardation	10/41	24
Seizures	47/49	96
Prenatal growth delay	25/26	96
Postnatal growth delay	43/50	86
Microcephaly	44/47	94
Hypotonia	17/19	89
Congenital heart defects	1/47	2
Cleft lip/palate	4/49	8
Ocular colobomas	0/29	0
Hypospadias	6/10	60
Renal abnormalities	1/42	2
Skeletal anomalies	8/29	28

Gandelman et al. [1992], Johnson et al. [1994], Clemens et al. [1996], Lindeman-Kusse et al. [1996], Fang et al. [1997], Partington et al. [1997], Wieczorek et al. [2000a], Maas et al. [2007], South et al. [2008a], Zollino et al. [Zollino et al., 2000, Zollino et al., 2003, Zollino et al., 2004, Zollino et al., 2007].

parental origin (Table V). A double cryptic chromosome imbalance turns out to be an important factor to explain phenotypic variability in WHS. The presence of the extra chromosome material on the deleted 4p can cause large 4p deletions to be mistaken as very small deletions, whenever the molecular diagnosis is carried out with a single molecular probe falling within the critical region. More importantly, this situation can mislead genotype–phenotype correlation analyses, since a double chromosome imbalance affect the degree of mental retardation, most likely.

TABLE II. Clinical Signs Associated With 4p Deletions of an Average Size Between 5 and 18 Mb (Our Patients: 48; Literature Patients: 58)

	No.	%
Typical facial dysmorphisms	106/106	100
Mild/moderate mental retardation	9/37	24
Severe mental retardation	28/37	76
Seizures[a]	61/76	80
Prenatal growth delay	26/31	84
Postnatal growth delay	79/87	91
Microcephaly	72/76	95
Hypotonia	39/43	91
Congenital heart defects	54/103	52
Cleft lip/palate	25/102	25
Ocular coloboma	30/101	30
Hypospadias	12/29	41
Renal abnormalities	31/83	37
Skeletal anomalies	23/58	37

Battaglia et al. [1999], Wieczorek et al. [2000a], South et al. [2008a], Maas et al. [2007], Zollino et al. [Zollino et al., 2000, Zollino et al., 2004, Zollino et al., 2007].
[a]EEG is not specified in several cases with apparently no seizures.

A high prevalence of t(4;8)(p16;p23) de novo translocations is reported in several large series of patients [Wieczorek et al., 2000b; Zollino et al., 2004, 2007; Maas et al., 2007; South et al., 2008a]. It is worth noting that two different breakpoints are recurrent on 4p, at about 9 Mb or, less frequently, 4.5 Mb from the telomere, respectively. These breakpoints correspond to the loci of two different olfactory receptor gene clusters. According to the different extent of the 4p deletion, the resulting phenotype differs significantly with respect to both severity of mental retardation and constellation of major malformations [Tonnies et al., 2001; Zollino et al., 2004]. However it must be specified that the 4p breakpoint in t(4;8) seldom occurs outside the ORs (Table VI, patient 52 in Zollino et al. [2007]).

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With the exception of the partial 8p trisomies, that, being OR-mediated [Giglio et al., 2002] usually span about 8 Mb, the remaining associated trisomies in unbalanced translocations are of different extent, varying from several megabases to few kilobases (Table VII).

On average, the associated trisomies do not affect significantly the physical phenotype. However further genotype–phenotype correlation analyses and follow-up are needed to better clarify this point.

MECHANISMS OF ORIGIN AND FAMILIAL RECURRENCE

Among the heterogeneous categories of the WHS-associated rearrangements, t(4p;8p) translocations represent a distinct genetic entity. They usually arise in the maternal meiosis as a result of homolo-

TABLE III. Clinical Signs Associated With Deletions >22–25 Mb (Our Patients: 4; Literature Patients: 6)

	No.	%
Typical facial dysmorphisms, not consistent with WHS	10/10	100
Mild/moderate mental retardation	0/5	0
Severe mental retardation with absent speech and walking	5/5	100
Seizures	9/10	90
Prenatal growth delay	8/8	100
Postnatal growth delay	7/9	78
Microcephaly	9/9	100
Hypotonia	7/7	100
Congenital heart defects	7/10	70
Cleft lip/palate	4/9	44
Ocular defects (coloboma)	8/10	80
Hypospadias	3/8	38
Renal abnormalities	6/16	38
Skeletal anomalies	6/18	33
Psychotic behavior	2/4	50

Wieczorek et al. [2000a], Maas et al. [2007], South et al. [2008a], Zollino et al. [Zollino et al., 2000, Zollino et al., 2007].

TABLE V. Parental Origin of De Novo 4p Rearrangements [Zollino et al., 2007]

Maternal	10/45 (22%)
t(4p;8p)	5
t(4p;7p)	1
t(4p;20q)	1
del(4p) interstitial	1
del(4p) terminal[a]	2
Paternal	35/45 (78%)
Del(4p) terminal	30
Del(4p) interstitial	2
t(4p;11p)	1
t(4p;acro p-arm)	1
der(4)(4qter → q32:: 4p15.3 → qter)	1

[a]A 1.4 Mb deletion was detected in the mother of one patient.

gous non-alleleic recombination mediated by olfactory receptor gene clusters on both 4p and 8p, and, in addition, can be associated with a maternal inversion polymorphism on 4p16 [Giglio et al., 2002; Zollino et al., 2007]. Consistent with all these elements, the clinical entity

Among the heterogeneous categories of the WHS-associated rearrangements, t(4p;8p) translocations represent a distinct genetic entity. They usually arise in the maternal meiosis as a result of homologous non-alleleic recombination mediated by olfactory receptor gene clusters on both 4p and 8p, and, in addition, can be associated with a maternal inversion polymorphism on 4p16.

TABLE IV. 4p De Novo Rearrangements in 54 Patients Studied With all Telomeres and/or Locus-Specific FISH, and/or a-CGH

Type of the rearrangement (Tot 54)	No.	%
Isolated deletions	38	70
Unbalanced translocations	12	22
t(4p;8p) (7)		
t(4p;7p)		
t(4p;11p)		
t(4p;12p)		
t(4p;20q)		
t(4p;Dp/Gp)		
Dup/del 4p	3	6
der(4)(4qter → q32::4p15.3 → qter)	1	2

resulting from t(4p;8p) translocations can be considered a genomic disorder.

The remaining WHS-associated rearrangements, including other (4p; autosomal) unbalanced translocations arise from different, yet unknown, mechanisms. They usually originate in the paternal meiosis and are neither related to a parental inversion polymorphism on 4p, nor OR-mediated [Zollino et al., 2007; Maas et al., 2007; Tables VI and VIII in the present report]. A great variability in the extent of individual deletions is observed in these cases, giving rise to phenotypic variability (Tables VI and VIII).

Provided that both parents had normal chromosomes, familal recurrence of WHS has never been reported, suggesting that germline mosaicism is uncommon in this condition, and that 4p inversion polymorphism cannot be regarded as a significant risk factor for meiotic rearrangements. Actually, the only instance of familial recurrence of a pure 4p deletion in two brothers was a

TABLE VI. Molecular Characterization of 22 Unbalanced Translocations, Either Familial or De Novo

	Locus	Probes	Distance from 4pter (Mb)	30	39	40	48	140	152	52	74	145	87	127	43	148	24	81	116	151	7	149	118	25	50
				t(4;8)(p16.1;p23)dn	t(4;8)(p16.1;p23)dn	t(4;8)(p16.1;p23)dn	t(4;8)(p16.1;p23) pat	t(4;8)(p16.1;p23)dn	t(4;8)(p16.1;p23)dn	t(4;8) (p16.3;p23)dn	t(4;8) (p16.3;p23)dn	t(3 ;4)(p26.1;p15.33)mat	t(4;7)(p16.3;p23)dn	t(4;7)(p16.3;p22.2)mat	t(4;10) (p16.3;p15)mat	t(4;10)(q26.11;p15.2)mat	t(4;11)(p16.1;p15.5)dn	t(4;11)(p16.1;q25) mat	t(4;11)(p16.1;q23.3)pat	t(4;12)(p16.3;p13.33)dn	t(4;20)(p16.2;q13.3)dn	t(4;21)(p16.1;q22.2)mat	t(4;D/G)(p16.2;p11)dn	t(4;13) (p16.1;p11) mat	t(4;13) (p16.;q33) mat
	S3359	Tel		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	S90	CD2																							
	S96	pC678		-		-				-		-	-	-	-	-			-	-			-	-	-
	S98 FGFR3	pC385.12	1,8											-											
WHCR2	S3327	190b4	1,95	-	-		-	-	-								-		-		-	-		-	
WHCR		19h1	2																						
	S43	33c6	2,3		-		-								-									-	
	S182	247f6	2,9																				-		
	S180	21f12	3,4												-					-					
OR	S81	**228a7**	3,7								-		-		+		-			+					
		RP11-324I10	4,25								+	-	+												
	MSX1	MSX-1	4,9	-		-	-		-	-	+		+	-	+		-			+			-	-	-
		RP11-524D9	5,9										+	+							-		-		
		RP11-367J11	7,2	-	-		-			+				+					-		+		+		
		RP11-358C18	8,6	-	-	-	-	-						+			-	-	+		+			-	-
OR		**RP11-423D16**	9	-	-	-	-	-	-			-				-	+					-		-	
		RP11-751L19	9.4	+	+	+	+	+	+							-	+					-		-	
		RP-17I9	9.7																			-			
		RP11-270I3	10.8																			+			
		RP11-3M2	12.6									-													
		RP11-731E20	13	+			+					+						-						-	-
		RP11-358H2	14.1															+						+	-
		RP11-81L15	14.6																					+	-
		RP11-565F20	15.4	+	+	+	+	+	+			+				-	+					+		+	+
		RP11-1J22														-									
		RP11-420H4														+									

Individual deleted probes on 4p are shown. Probes delimiting the proximal and distal OR are indicated (bold). The shaded area represents the extent of the deletions. This table is updated (six more patients) with respect to that reported in Zollino et al. [2007].

The breakpoint on 4p occurred at the proximal OR (probe RP11-423D16 deleted, probe RP11-751L19 preserved) in six subjects, carrying a t(4p;8p) translocation (no. 30, 39, 40 and 48, 140 and 152). It was within the distal OR (probe 228a7 deleted, probe RP11-324I10 preserved) in two subjects, with a t(4p;8p) and a t(4p;7p) translocation, respectively (no. 74 and 87).

Please note that in patient 52 the t(4p;8p) is not OR-mediated on 4p.

TABLE VII. Complex Rearrangements: Extent of the Associated Trisomies in Both De Novo and Parental Anomalies

Type of rearrangement	4p deletion (Mb)	Patient no.	Associated trisomy
t(4;8)(p16.1;p23)dn	9.2	30	8p (8 Mb)
t(4;8)(p16.1;p23)dn	9.2	39	8p (8 Mb)
t(4;8)(p16.1;p23)dn	9.2	40	8p (8 Mb)
t(4;8)(p16.1;p23)dn	9.2	140	8p (8 Mb)
t(4;8)(p16.1;p23)dn	9.2	152	8p (8 Mb)
t(4;8)(p16.3;p23)dn	5.3	52	8p (8 Mb)
t(4;8)(p16.3;p23)dn	4.3	74	8p (8 Mb)
t(4;11)(p16.1;p15.5)dn	8.8	24	11p (0.8 Mb)
t(4;7)(p16.3;p22)dn	4.3	87	7p (6.5 Mb)
t(4;12)(p16.3;p13.33)dn	3.5	151	12p (380 kb)
t(4;20)(p16.2;p13.3)dn	6	7	20q (6 Mb)
t(4;Dp/Gp)(p16.2;p11)dn	6	118	acro p arm
dup/del 4p (dn)	2.5	14	4p (8 Mb)
dup/del 4p (dn)	4.3	20	4p (15 Mb)
dup/del 4p (dn)	6.5	22	4p (18 Mb)
der(4)(4qter → q32::4p15.3 → qter)dn	18	45	4q (18 Mb)
t(4;7)(p16.3;p22.2)mat	5.3	127	7p (4.3 Mb)
t(4;10)(p16.3;p15)mat	3.5	43	10p (4 Mb)
t(4;11)(p16.1;q25)mat	13.5	81	11q (15 Mb)
t(4;13)(p16.1;p11)mat	13.5	25	13p (n.a.)
t(4;13)(p16.1;q33)mat	15	50	13q (10 Mb)
t(4;8)(p16.1;p23.1)pat	9.2	48	8p (8 Mb)
t(4;11)(p16.1;q23.3)pat	8	116	11q (15 Mb)
t(3;4)(p26.1;p15.33)mat	12.8	145	3p (4.2 Mb)
t(4;10)(q26.11;p15.2)mat	23.4	148	10q (17 Mb)
t(4;21)(p16.1;q22.2)mat	10.2	149	21q (n.a.)
t(4;12)(p16.3;p13.3)mat	1.6	CC[a]	12p (6 Mb)

ORs on 4p are localized at about 9.2 and 4.3 Mb from the telomere.
n.a., not available.
[a]The patient is not affected by WHS.

meiotic amplification of a maternal small deletion [Faravelli et al., 2007].

PHENOTYPIC MAP

Material and Methods

Clinical genetic data of 73 WHS were reported previously [Zollino et al., 2000, 2003, 2007; Rodriguez et al., 2005]. Patient MT was already described by Faravelli et al. [2007]. This patient has now been further investigated with additional molecular probes, to narrow the proximal breakpoint of the deletion.

Detailed clinical and genetic data are provided only for WHS patients not previously reported (n = 7), and for one patient without WHS in which a der (4)(4qter → p16.3::12p13.3 → qter) was detected. Some special comments are deserved to this last patient. The 4p deletion included the terminal 1.6 Mb region, thus both *WHSC1* and *LETM1* were preserved. Although the associated 12p trisomy, spanning about 6 Mb, is likely to affect the final phenotype, it is worth noting that this patient presented with accelerated growth from early on, no typical facial dysmorphisms, and, in addition, she had seizures. All these considerations led us to restrict the distal boundary for growth delay in WHS at 1.6 Mb from the telomere on 4p, as well as to make a confirmation that other genes, in addition to *LETM1*, distal to it, are related to the seizure disorder.

In the majority of patients, molecular karyotyping was carried out by FISH with a total of 92 locus-specific molecular probes, that were selected in individual patients to form a contig, whenever possible, encompassing the breakpoint regions. A list of these and their chromosome localization were already reported [Zollino et al., 2007]. The associated trisomies were characterized by means of locus-specific FISH. In addition to the molecular probes reported in Zollino et al. [2007], in newly diagnosed patients were tested the following: 3p-specific (3p telomere (Vysis, Downers Grove, IL); PR11-453F3; RP11-250A15; RP11-140B10; RP11-124L4); 10q-specific (10q telomere (Vysis); RP11-245J24; RP11-498B4). Two patients (n 151 and 152 in the present report) underwent array-CGH analysis at a resolution of 75 kb (Kit 44B, Agilent Technologies, Santa Clara, CA).

Clinical and genetic data of the newly reported patients are summarized in Table IX.

Proximal breakpoint of the 4p deletion in patient MT, already reported by Faravelli et al. [2007] was redefined by FISH to fall between probe RP11-386I15 (deleted) and RP11-1398P2 (preserved), being the resulting deletion of about 1.4 Mb.

A comparative analysis of all patients (n = 80) with WHS and patients (n = 2) with a small terminal deletion not productive of WHS allowed us to define a phenotypic map for the distinctive WHS clinical signs (Fig. 4).

Included in this genotype–phenotype correlation analysis was one patient in which an interstitial 4p deletion not productive of WHS was detected, with distal breakpoint at 9 Mb from the telomere. This patient presented with accelerated growth and hypospadias. He will be described in detail in a separate report, along with additional 4p interstitial deletions.

We found that epilepsy map on the terminal 1.9 Mb region and typical facial dysmorphisms on a 300 kb interval comprised between 1.9 and 1.6 Mb

TABLE VIII. Molecular Characterization of Individual Chromosome Anomalies, Other Than Unbalanced Translocations. Probes Delimiting Proximal and Distal OR on 4p are Indicated

	Probes	Distance from 4pter (Mb)	Patient																															
			37	1	80	2 3 4 5 36 41 45° 89	18	42	73 128	139	32 78 82	112	111	33	131	59	8 9 85 88 91 119	6 11 22* 117 146	10 35 120	31 86	92	20*	124	44	98 **	12	13	26 46 77 103 115	14*	15 75 141	MG 97
	pC847.351	0,15	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	+		-	-	-	-	-	-	-	-	-	-
	CD2	0,25																			+													
	pC678	1,2	-	-			-	-		-	-			-	-	-	-				-		-	-					-	-		-
	pC385.12	1,8																												-		-
WHCR2	**190b4**	**1,95**		-		-		-		-		-	-		-				-	-				-	-	-	-		-	-	-
WHCR	**19h1**	**2**																													-	+
	108f12	2,2										-					-												-		-	+
	33c6	2,3	-	-		-		-						-					-	-			-	-		-	-	-	-	-	+	
	161c2	2,35																									-					
	79f5	2,5																									-		-			
	212a9	2,5																											+			
	185f6	2,6																								-	-					
	247f6	2,9																		-				-	-	-	+	+	+			
	21f12	3,4															-			-				-		+	+	+				
OR	**228a7**	**3,7**																		-		-	-	+	+	+						
	RP11-324I10	**4,25**																		-	-		+									
	RP11-368B4	4,3																		-	-		+									
	MSX1	4,9		-			-		-		-	-	-	-	-		-		-	+	+	+	+		+	+		+				
	RP11-524D9	5,9																-	+													
	RP11-367J11	7,2				-						-				-	-	+	+													
	RP11-358C18	8,6						-	-			-	-	-		-	+		+													
OR	**RP11-423D16**	**9**										-	-		-	+		+														
	RP11-751L19	**9,4**									-	-	-	-	+	+		+	+														
	RP11-17I9	9,7									-			-																			
	RP11-270I3	10,8							-		-			+																			
	RP11-1J7	11,6								-	+		+	+	+				+														
	RP11-3M2	12,6							-	+	+		+																				
	RP11-731E20	13						-	-	+			+																				
	RP11-356H2	14,1						-	+	+																							
	RP11-81L15	14,6						+	+																								
	RP11-469O6	15				-	-										+																
	RP11-565F20	15,4				-	+	+	+	+	+	+	+	+	+																		
	RP11-332D24	20			-	+											+																
	RP11-481K22	22		-		+																											
	RP11-778B12	25,4		+	+																												
	RP11-497E20	27	-		+																												
	RP11-390C19	29,5	+	+																													

The shaded area represents the extent of the deletions.

The distal OR was demonstrated to mediate only one isolated terminal deletion (case no. 124); it could be involved also in one case with dup/del 4p (no. 20). The proximal OR mediated only one isolated terminal deletion (case no. 131).

*dup/del 4p ** ring(4) ° der(4)(4qter → q32::4p15.3 → qter). All the remaining cases are isolated deletions (2 interstitial and 50 terminal).

All the patients, but one (Pat 141) were previously reported in Zollino et al. [2000, 2003, 2004, 2007], Rodriguez et al. [2005].

TABLE IX. Clinical Data of the 8 WHS Patients Here Reported for the First Time

	CC* (F 4 ys)	140 (F 4ys)	141 (F 12 ys)	145 (F 2 ys)	148 (F 13 ys)	149 (F 17 ys)	151 (F 9 ys)	152 (F 12 ys)
Typical facial dysmorphisms	−	+	+	+	+	+	+	+
MR	+	+	+	+	+	+	+	+
Seizures	+	n.a.	n.a.		+	+	+[a]	+
Growth delay	Overgrowth	+	+	+	+	+	+	+
Microcephaly	Macrocephaly	+	n.a.	+	+	+	+	+
Hypotonia	+	+	n.a.	+	+	+	+	n.a.
CHD	n.a.	n.a.	n.a.		VSD	n.a.	−	ASD
Cleft lip/palate	−	n.a.	n.a.		+	n.a.	−	−
Ocular defects	−	n.a.	n.a.		Iris coloboma	n.a.	−	Iris coloboma
Renal abnormalities	n.a.	n.a.	n.a.		−	n.a.	−	+
Type of rearrangement	t(4p;12p)mat	t(4p;8p)dn	Isolated deletion	t(4p;3p)mat	t(4p;10q)mat	t(4p;21q)mat	t(4p;12p)dn	t(4p;8p)dn
4p deletion size	1.6	9.2	2.3	12.8	23.4	10.2	3.5[b]	8.7[b]

[a]EEG anomalies only.
[b]Array-CGH.
*The patient is not affected by WHS.

from the telomere. Additional genes for less penetrant facial phenotype are likely to reside distally to this interval, as here discussed in Pathogenesis of WHS Section. A very restricted region for growth delay was defined, limited to about 300 kb between 1.9 (patient MG in Zollino et al. [2003]) and 1.6 (patient CC in the present report) Mb from the telomere. Microcephaly had distal boundary at 2.2 Mb from the telomere, and cleft palate at 2.5 Mb. Chromosome localization of additional clinical signs can be easily inferred from the same figure.

Our conclusions are largely in agreement with Maas et al.'s [2008] suggestions, with a few modifications.

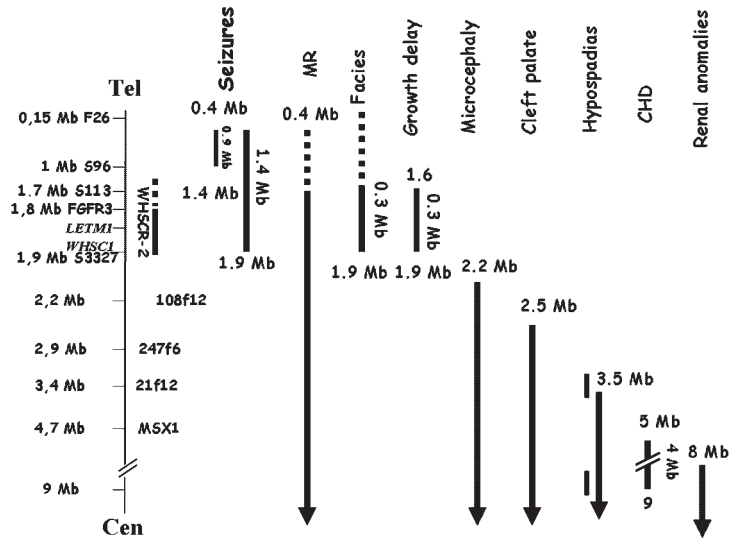

Figure 4. Physical mapping of the distinctive clinical signs in WHS. Relevant for these considerations was a patient with an interstitial 4p deletion, with distal breakpoint at 9 Mb from the telomere, who presented with overgrowth and hypospadias. This patient in not included in the present report. With respect to mental retardation, the dotted line refers to very mild mental retardation. Distal breakpoint for mental retardation and seizures was established at about 0.4 Mb from the telomere on the basis of van Buggenhout et al.'s [2004] report. Two different loci were noted for both "seizures" (Faravelli et al.'s report) and "hypospadias" (the patient with interstitial 4p deletion preserving the terminal 9 Mb and presenting with hypospadias).

PATHOGENESIS OF WHS

After the initial hypothesis that a single gene could be responsible for the whole WHS phenotype, the pathogenesis of WHS is considered to be multigenic [Bergemann et al., 2005]. Firstly, we consider that critical genes responsible for the core phenotype all reside within the terminal 1.9 Mb interval, *WHSC1* being the most proximal. It was suggested that haploinsufficiency of *WHSC1* causes the facial characteristics and growth delay, while hemizygosity for *LETM1* is responsible for the seizure disorder [Maas et al., 2008]. We agree with these conclusions, but additional considerations are in order. The patient reported by Rauch et al. [2001] had a small interstitial deletion limited to WHSCR, causing hemizygosity for *WHSC1* and preserving *LETM1*. This patient presented with a thin habitus, normal height, and with some subtle facial characteristics consistent with WHS. More importantly, he never had seizures. The patient reported by

Faravelli et al. [2007], here further analyzed molecularly, carries a 1.4 Mb deletion on 4p preserving both *WHSC1* and *LETM1*. Growth was normal in infancy, although final stature was relatively short. She presented with borderline mental retardation and had seizures as a child. In addition, some ocular and nasal characteristics consistent with WHS were noted with ageing. Based on this evidence, we can assume that, in addition to *WHSC1*, other genes distal to it, although less penetrant, are responsible for both facial characteristics, and growth delay. It should be specified that the pattern of growth delay in WHS is usually severe, being of prenatal onset, and affecting both height and weight. Thus, WHSC1 and other genes have a cumulative effect on growth delay, most likely.

Also relevant with respect to genes responsible for seizures, are two typical WHS patients with a 1.9 Mb terminal deletion encompassing *LETM1* and seizures [Zollino et al., 2003; Rodriguez et al., 2005]. We can conclude that seizures in WHS are likely caused by hemizygosity not only of *LETM1*, but of other gene as well, residing within the terminal 1.9 Mb region of chromosome 4p [South et al., 2008b].

We can conclude that seizures in WHS are likely caused by hemizygosity not only of LETM1, but of other gene as well, residing within the terminal 1.9 Mb region of chromosome 4p.

With respect to growth delay, literature reports provide contradictory data. A small 4p region for growth retardation was recently identified by two independent groups within the terminal 760 kb [Concolino et al., 2007] and the terminal 1.27–1.46 Mb [South et al., 2007], respectively. However this evidence came in both cases from a ring chromosome 4, making its interpretation difficult. It is known that delayed growth is a nonspecific manifestation of ring syndromes of any type [Kosztolànyi, 1987; Zollino et al., 2008]. Against the evidence that growth delay maps within these regions is the 1.4 Mb deleted patient reported by Faravelli et al. [2007], and patient CC in the present report. Although the about 6 Mb 12p trisomy detected in this patient in association with the 1.6 Mb 4p deletion may affect the final phenotype, growth parameters were within normal upper limits.

These observations prompted us to restrict the chromosome interval for growth delay to a 300 kb region between 1.9 Mb (cosmid 190b4 included) and 1.6 Mb (BAC RP11-572O17 included) from the telomere. We conclude that haploinsufficiency not only of *WHSC1*, but also of additional genes, residing within this interval, is required for the severe growth delay usually observed in WHS.

Prognostic Factor for Mental Retardation

Beside the extent of the 4p deletion, and the occurrence of a double chromosome imbalance, that are well established prognostic factors in WHS, seizures are also an independent prognostic factor for degree of mental retardation [Battaglia et al., 1999]. Two patients with a very small deletion, of 2.2 and 2.6 Mb, respectively [patient 15 in Zollino et al., 2000, 2003 and patient 46 in Zollino et al., 2007], predictive of a mild phenotype, had severe mental retardation with nearly absent language, and both suffered from a severe epileptic status in the early infancy. Patient 13 in the Zollino et al. [2007] report, carrying a pure 2.6 Mb 4p deletion, and patient 151 in the present report, in which a 3.5 Mb 4p deletion was detected in association with a 12p trisomy of 0.4 Mb in length, both presented with a very mild mental retardation. They can easily write and read, they can play piano, and they are independent in daily activities. Language is rich and fluent. They never had overt seizures, but only EEG anomalies as a child. Patient 151 will be described in detail in a future paper.

FINAL CONSIDERATIONS

Our observations indicate that the etiology of WHS is multigenic, with different genes involved in the production of distinctive clinical signs. Genomic rearrangements productive of the core WHS phenotype are expected to be chromosome deletions event in all cases, and no typical WHS patients are expected to harbor a single gene mutation. However, among the recognized far characterized candidate genes, *WHSC1* is likely to be a developmental gene, whose loss-of-function mutations could result in a constellation of clinical signs resembling WHS. According to all previous considerations, mutational analysis of *WHSC1* should be considered only for those non-deleted patients presenting with facial characteristics resembling WHS, mild mental retardation and mild growth delay, but with no seizures. Since congenital malformations have been all related to contiguous deletions, proximal to WHSCR-2, patients with malformations, even falling within the WHS phenotype, should not be included in the search for *WHSC1* mutations. Some non-deleted patients were already screened for *WHSC1* mutations by Stec et al. [1998] and Maas et al. [2008], with negative results. However phenotypic information was not provided in those reports.

Some further considerations are in order. Severe growth delay and seizures represent the greatest problems in the clinical management of WHS. Characterizing the pathogenic genes for these signs could allow the design of a gene therapy for WHS. Given that haploinsufficiency is the basic pathogenetic mechanism in WHS, the unaltered copies of each deleted gene on the homologous chromosome are the ideal target for attempts at enhancing their expression by reactivating drugs.

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