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ORIGINAL INVESTIGATION

Wolf–Hirschhorn syndrome-associated chromosome changes are not mediated by olfactory receptor gene clusters nor by inversion polymorphism on 4p16

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Abstract The basic genomic defect in Wolf–Hirschhorn syndrome (WHS), including isolated 4p deletions and various unbalanced de novo 4p;autosomal translocations and above all t(4p;8p), is heterogeneous. Olfactory receptor gene clusters (ORs) on 4p were demonstrated to mediate a group of WHS-associated t(4p;8p)dn translocations. The breakpoint of a 4-Mb isolated deletion was also recently reported to fall within the most distal OR. However, it is still unknown whether ORs mediate all 4p-autosomal translocations, or whether they are involved in the origin of isolated 4p deletions. Another unanswered question is whether a parental inversion polymorphism on 4p16 can act as predisposing factor in the origin of WHS-associated rearrangements. We investigated the involvement of the ORs in the origin of 73 WHS-associated rearrangements. No hotspots for rearrangements were detected. Breakpoints on 4p occurred within the proximal or the distal olfactory receptor gene cluster in 8 of 73 rearrangements (11%). These were five t(4p;8p) translocations, one t(4p;7p) translocation and two isolated terminal deletions. ORs were not involved in

Web Resources: Electronic Database Information: Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for WHS [MIM 194190]; Ensembl Human Map, http://www.ensembl.org; UCSC, http://genome.ucsc.edu.

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one additional t(4p;8p) translocation, in a total of nine different 4p;autosomal translocations and in the majority of isolated deletions. The presence of a parental inversion polymorphism on 4p was investigated in 30 families in which the 4p rearrangements, all de novo, were tested for parental origin (7 were maternal and 23 paternal). It was detected only in the mothers of 3 t(4p;8p) cases. We conclude that WHS-associated chromosome changes are not usually mediated by low copy repeats. The 4p16.3 inversion polymorphism is not a risk factor for their origin.

Introduction

Wolf–Hirschhorn syndrome (WHS) [MIM 194190] is a multiple congenital anomalies/mental retardation (MCA/MR) syndrome caused by deletion of the distal region of chromosome 4p (Wolf et al. 1965; Hirschhorn et al. 1965). The critically deleted region, WHSCR-2, was recently mapped to a 300–600 kb interval in 4p16.3, between loci D4S3327 and D4S98–D4S168 (Zollino et al. 2003). It lies contiguously distal to the previously described critical region (WHSCR) (Wright et al. 1997), sharing with it the candidate gene *WHSC-1*.

The majority of WHS-associated genomic rearrangements are de novo events, assumed to be isolated deletions. However, an increasing number of unbalanced translocations were recently described, usually consisting of t(4p;8p)dn translocations (Wieczorek et al. 2000; Zollino et al. 2004; Tonnies et al. 2001; Petit et al. 1998; Muller-Navia et al. 1996) involving the olfactory gene cluster regions (ORs) (Giglio et al. 2002). The involvement of the distal OR on 4p was recently reported in one isolated deletion (Van Buggenhout et al. 2004). However, it is still unknown whether ORs mediate all 4p-autosomal translocations, and how frequently they are involved in the origin of isolated 4p deletions.

All the so far reported unbalanced de novo translocations were maternal in origin (Wieczorek et al. 2000; Zollino et al. 2004; Giglio et al. 2002). However, no reports deal with a comparative analysis of prevalence, parental origin, and mechanism of origin of different WHS-associated rearrangements.

Heterozygous cryptic inversions involving ORs were demonstrated to mediate the recurrent t(4:8)(p16;p23) translocation in WHS (Giglio et al. 2002), suggesting that a parental inversion polymorphism on 4p can act as predisposing factor in the origin of WHS-associated rearrangements, but no conclusive data exist in the literature. We tried to clarify these points by analysing a large cohort of WHS subjects and families.

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Subjects and methods

A total of 73 WHS subjects; 33 males and 40 females, aged between 8 months and 24 years, were analysed both clinically and genetically. A summary of the clinical data is available on Table 1 online.

Genetic analysis was carried out on peripheral blood cell with the following techniques: (1) R(RBG) banding at a resolution of 550 bands; (2) FISH analysis on metaphase chromosomes. Probes 228a7 and RP11-324I10, delimiting the distal OR on 4p, and probes RP11-423D16 and RP11-751L19, delimiting the proximal OR, were tested in each patient. Another 33 molecular probes, spanning the 4p15pter chromosome region, properly selected for use in individual patients, were tested to assess the individual 4p deletion size. Molecular probes were chosen to form a contig for the terminal 5 Mb region, and they were set up at 200–500 kb intervals for the proximal regions. (3) FISH analysis of subtelomeric regions in 47 cases with de novo rearrangements, to look for unbalanced de novo translocations; (4) FISH analysis with locus-specific FISH probes in three patients with dup/del 4p, to characterise the associated 4p proximal duplication; (5) locus-specific FISH for different chromosome regions (in the remaining 16 patients with double rearrangements), to characterise the associated trisomy; (6) microsatellite segregation analysis, to ascertain the parental origin of the rearrangement.

Parents were analysed in all cases, by means of both conventional cytogenetics and FISH with two molecular probes, the first one falling within the WHSCR-2 region, the second detecting the 4p subtelomeric region. A total of 25–200 metaphases were scored in each parent. Lymphoblastoid cell lines were established from 44 patients and their parents.

FISH probes specific for the 4p arm

Molecular probes specific for the 4p arm included 16 cosmid clones, 21 BAC clones belonging to RPCI11 library

and the subtelomeric 4p probe provided by Vysis. They are listed in Tables 2 and 4.

All patients were investigated with the 4p subtelomeric probe provided by Vysis, and with probes pC847.351 (subtelomeric), 190b4 (falling within the newly described critical region, WHSCR-2) and 19h1 (falling within the first described critical region, WHSCR). The remaining probes were properly selected to be tested in each subject by multistep FISH analysis, as suggested by the results of the conventional cytogenetics. A 500-kb resolution FISH was first performed to look for the breakpoint region that was then covered by a contig. Probes were chosen from libraries available on the web (UCSC, ncbi, ensembl). Most of them were kindly provided by T. Wright and M. Rocchi. Probes were labelled with biotin or digoxigenin and detected by fluorescein-conjugated avidin or fluorescent anti-digoxigenin antibodies, or they were directly labelled with Spectrum Orange and/or Spectrum Green (Vysis). DAPI counterstain was applied. Metaphases were visualised using a Zeiss Axioplan 2 imaging fluorescent microscope equipped with single-band pass filters. Digital images were captured and analysed with the ISIS program (Meta Systems). At least 20 metaphases were analysed by direct microscopic observation and digital-imaging techniques.

Subtelomeric FISH

All subtelomeric regions were analysed on metaphase chromosomes from 47 WHS patients using the ToTelVysion™ Probes (Vysis).

Metaphases of patients carrying a derivative chromosome 4 were also analysed by Whole Chromosome Paint probes (Vysis) and by locus-specific probes, to assess the extension of the trisomic segment.

Locus-specific FISH probes

Double rearrangements were further characterised with the following probes:

- 8p-specific: RP11-372K15, RP11-403C10;
- 7p-specific: RP11-449P15, RP11-42B7, RP11-160E17, RP11-527E14, RP11-425P5;
- 11p-specific: RP11-745B17, RP11-401C19, RP11-295K3;
- 11q-specific: RP11-861M13, RP11-730K11, RP11-791J7;
- 4p-specific YACs (for inv dup/del): 725B6, 815H5, 758E8, 657E4, 819F7, 776G9, 758G12, 787C10, 850C11
- 20-specific: wcp, RP11-732I1

Microsatellite segregation analysis

Parental origin of de novo rearrangements was investigated in a total of 45 families. Genomic DNA was prepared by standard protocols from peripheral blood lymphocytes or from lymphoblastoid cell lines of patients and parents.

Microsatellites were D4S3360, D4S2936, D4S3038, D4S1614, D4S127, D4S3034, D4S412 (Eurobio), all mapping within 4p16. PCR products were electrophoresed in 6% denaturing polyacrylamide gels and were visualised by autoradiography (Shaffer et al. 1993) or by silver staining.

Inversion of the 4p16 region flanked between the distal and the proximal OR

The presence of the inversion polymorphism affecting the about 5 Mb region flanked by two ORs on 4p16 was checked by interphase dual-colour FISH. FISH experiments were carried out with probes RP11-690D17 and RP11-323F5, falling within this region, and probe RP11-520M5, lying outside and distally to it (Fig. 1). A total of 30 families were analysed.

Results

Characterization of the 4p deletion by molecular cytogenetics

In all of our patients the 4p deletion included WHSCR-2 (Zollino et al. 2003). WHSCR was also deleted in all

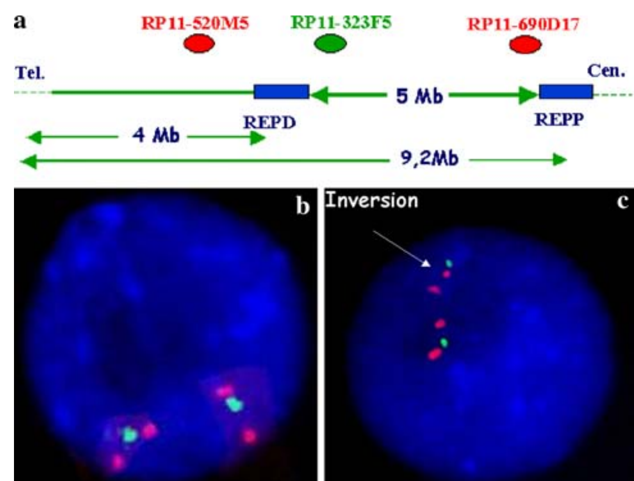

Fig. 1 Dual-colour interphase FISH looking at the 4p16 inversion polymorphism. Probe RP11-520M5 and RP11-690D17 are labelled with *spectrumOrange* (Vysis), probe RP11-323F5 is labelled with *spectrumGreen* (Vysis) (**a**). *Red–green–red* means absence of inversion (**b**). *Red–red–green* means presence of inversion (**c**). Fifty to hundred nuclei were analysed in individual subject

patients except two (MG and patient 97), as previously reported (Zollino et al. 2003; Rodriguez et al. 2005).

The partial 4p monosomy was terminal in 71 patients (97%), and interstitial in two (3%) with distal breakpoint at a distance of about 250 kb from the telomere. A great variability of the 4p deletion size was observed, ranging from 1.9 to 29 Mb, as shown in Tables 2 and 4.

Molecular characterization of the basic genomic change

Seven patients (10%) presented a derivative chromosome 4 segregating from a balanced parental translocation. (The derivative chromosomes are listed in Table 2 online). The remaining 66 rearrangements (90%) were de novo.

When 50 de novo rearrangements were analysed by either subtelomeric probes (47 cases) or by locus-specific FISH probes (3 cases), we found that 37/50 (74%) were pure 4p deletions, 9 (18%) were unbalanced translocations, 3 (6%) were inverted 4p duplications associated with a terminal deletion on the same chromosome arm. In one case (2%) the genomic rearrangement consisted of a der(4)(4qter→q32::4p15.3→qter), resulting in partial 4q trisomy and partial 4p monosomy (Table 1). Breakpoints on 4p in WHS-associated unbalanced translocations are reported in Table 2.

Breakpoints on the partner chromosomes were established by locus-specific FISH analysis in 12 out of 14 informative cases (in patients 25 and 118 the partner region was the satellite of the short arm of one acrocentric chromosome). (These breakpoints and the corresponding size of the deletions and associated trisomies are listed in Tables 2 and 3 online.)

Parental origin of the rearrangement

De novo rearrangements occurred on the paternal chromosome in 35 of 45 patients (78%), and they were usually isolated deletions, with the exception of two unbalanced translocations, t(4p;11p) and t(4p;Dp/Gp) (Table 3). The above-mentioned der(4)(4qter→q32::4p15.3→qter) derivative chromosome also originated in the paternal meiosis. Rearrangements were of maternal origin in 10 of 45 patients (22%). Maternally derived anomalies were usually unbalanced translocations, particularly t(4p;8p) translocations (five cases), but also t(4p;7p) (one case) and t(4p;20q) (one case). Only two maternally derived anomalies were isolated terminal deletions (cases 12 and 115). A smaller 4p deletion, of about 1.5 Mb, spanning from the region identified by probe pC678 to the telomere and preserving the WHSCR-2, was detected in the mother of patient 12, whose older brother also had WHS, as previously reported (Faravelli et al. 2007). One maternally derived anomaly was an interstitial deletion. Breakpoints of different rearrangements, other than unbalanced translocations, are summarised in Table 4.

Table 1 4p de novo rearrangements in 50 patients studied with all telomeres and/or locus-specific FISH

Type of the rearrangement	No.	%
Isolated terminal deletion	37	74
Unbalanced translocations	9	18
t(4;8) (p16.1-p16.3;p23)	5	
t(4;7) (p16.1;p22)	1	
t(4;11) (p16.1;p15.5)	1	
t(4;20) (p16.2;q13.3)	1	
t(4;Dp/Gp) (p16.2;p11)	1	
Dup/del 4p	3	6
der(4)(4qter→q32::4p15.3→qter)	1	2

4p16 inversion polymorphism

This polymorphism was detected only in the mothers of three patients, always in association with t(4p;8p)dn translocations. In is worth noting that it was not detected in association with one additional maternally derived t(4p;8p), in one maternally derived t(4;7), in one paternally derived t(4;11), and in 23 isolated deletions, both paternal and maternal in origin.

The same inversion polymorphism was also observed in 4 fathers, but in a mosaic state 40–60% of nuclei), suggesting its origin in a post-zygotic event.

Discussion

WHS can be considered a contiguous gene syndrome with a basic phenotype defined by the association of mental retardation, typical facial appearance, growth delay and seizures (or EEG anomalies) (Zollino et al. 2003). Depending on the size of the deletion, additional clinical signs include microcephaly, congenital heart defects, midline anomalies, renal abnormalities and colobomas. The majority of WHS-associated chromosome changes are de novo, and they are expected to be isolated deletions. However, unbalanced de novo translocations, consisting more often of t(4p;8p) translocations, but also including a t(4p;7p) translocation, have been reported with unexpected high frequency in WHS, and they were all maternal in origin (Wieczorek et al. 2000; Zollino et al. 2004; Tonnies et al. 2001; Petit et al. 1998; Muller-Navia et al. 1996). These reports point at a great complexity of the WHS-associated chromosome defects, which can be the cause of the great phenotypic variability of this condition.

Table 2 Molecular characterization of 16 unbalanced translocations, either familial or de novo

	Locus	Probes	Distance from 4pter (Mb)	Patient															
				30	39	40	48	52	74	87	127	43	24	81	116	7	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) (p16.3;p23)dn	t(4;8) (p16.3;p23)dn	t(4;7)(p16.3;p23)dn	t(4;7)(p16.3;p22.2)mat	t(4;10) (p16.3;p15)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;20)(p16.2;q13.3)dn	t(4;D/G)(p16.2;p11)dn	t(4;13) (p16.1;p11) mat	t(4;13) (p16.1;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	+	+	+	+						+					-	
		RP11-731E20	13	+			+							-				-	-
		RP11-358H2	14.1											+				+	-
		RP11-81L15	14.6															+	-
		RP11-565F20	15.4	+	+	+	+						+					+	+

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. The breakpoint on 4p occurred at the proximal OR (probe RP11-423D16 deleted, probe RP11-751L19 preserved) in four subjects, carrying a t(4p;8p) translocation (nos. 30,39,40 and 48). 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 (nos. 74 and 87). Patients 30, 39, 40, 43, 52 and 74 were previously reported in Zollino et al. (2003, 2004). However, fine mapping of the breakpoints is presented here for the first time in most of them. Patient 7 was previously reported as having an isolated deletion (Zollino et al. 2000), but an unbalanced translocation was subsequently detected by all subtelomeric regions analysis

By analysing a large cohort of WHS patients, we found that the WHS-associated de novo rearrangements fall within the following four categories: pure terminal or interstitial deletions; de novo unbalanced translocations; proximal 4p inverted duplications associated with a terminal deletion on the same chromosome arm; and dup 4q/del 4p recombinant chromosomes (Table 1).

A strong relationship was observed between parental origin and type of the rearrangements. Paternally derived rearrangements, accounting for 78% of cases, were usually isolated terminal deletions, although interstitial deletions and complex rearrangements, including unbalanced translocations, were observed in a few cases (Table 3). Many WHS-associated rearrangements, all described as apparently isolated deletions, were reported to be paternal in origin by several authors (Quarrell et al. 1991; Tupler et al. 1992; Dallapiccola et al. 1993).

In our series of patients, 22% of de novo rearrangements originated in the maternal meiosis. They consisted usually of unbalanced translocations, more often t(4p;8p), but also

Table 3 Parental origin of de novo 4p rearrangements

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;Dp/Gp)	1
der(4)(4qter→q32::4p15.3→qter)	1

[a] A 1.5 Mb deletion, preserving the WHSCR-2, was detected in the mother of one patient (no. 12)

t(4p;7p) and t(4p;20q). Isolated deletions, both interstitial and terminal, were diagnosed in a few maternally derived cases (Table 3).

It can be reasonably inferred that specific parent of origin-dependent mechanisms exists in generating the WHS-associated chromosome changes, but the nature of this phenomenon is not clear.

Likewise, the question whether there exist chromosomal hotspots for rearrangement is still unanswered. Two ORs are present on the short arm of chromosome 4, at about 4 and 9 Mb from the telomere, respectively. Due to their relative homology, OR clusters can act as duplicons, and they can mediate intra and inter-chromosomal rearrangements (Giglio et al. 2002; Stankiewicz and Lupski 2002). It was recently demonstrated by Giglio et al. (2002) that the ORs on 4p mediate the WHS-associated t(4p;8p)dn translocations. Van Buggenhout et al. (2004), recently reported a 4-Mb terminal deletion with the breakpoint falling within the distal OR gene cluster. However, it is still unknown whether ORs mediate all 4p-autosomal translocations, or how frequently they can be responsible for isolated 4p deletions. We investigated these points in a total of 73 WHS families.

Looking at unbalanced translocations ($n = 16$), no hotspots were observed on 4p, with the exception of the t(4p;8p) translocations, that recurred within the proximal or the distal OR in six of seven cases. Only one different translocation, t(4p;7p)dn, did occur within the distal OR on 4p

Table 4 Molecular characterization of individual chromosome anomalies, other than unbalanced translocations

	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	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	+	+																											

Probes delimiting proximal and distal OR on 4p are indicated. 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) and ° der(4)(4qter→q32::4p15.3→qter). All the remaining cases are isolated deletions (2 interstitial and 50 terminal). Patients 1, 2, 3, 4, 5, 6, 8, 9, 10, 11, 12, 13, 14, 15, 26, 31, 32, 33, 35, 36, 37, 41, 42, 44, 45, 46, 73, 75, 77, 82, 97 and MG were previously reported in Zollino et al. (2000, 2003, 2004) and Rodriguez et al. (2005). However, fine mapping of the breakpoints is presented here for the first time in most of them

(patient 87). ORs were not involved in one additional t(4p;8p) translocation, and in a total of nine different 4p;autosomal translocations. In addition, we observed that ORs are only rarely involved either in isolated deletions, or in different WHS-associated complex rearrangements.

A second question that was addressed in the present study is whether the WHS-associated rearrangements can be related to the presence of a parental inversion polymorphism on 4p16.

Inversion polymorphisms have been related to the origin of the William-Beuren 7q11.23 deletion (Osborne et al. 2005) and of several additional chromosome imbalances (Giglio et al. 2001). In particular, heterozygous submicroscopic inversions involving ORs were shown to mediate the recurrent WHS-associated t(4;8)(p16;p23) translocation (Giglio et al. 2002). We detected the same 4p16 inversion only in three cases (5% of all cases analysed), and always in association with an unbalanced t(4p;8p) translocation. In is worth noting that it was not detected in association with one additional maternally derived t(4p;8p), in one maternally derived t(4;7), in one paternally derived t(4;11), and in 23 isolated deletions, both paternal and maternal in origin. The same inversion was observed in four fathers in a mosaic state, suggesting that this region undergoes mitotic instability.

We conclude that, with the exception of t(4p;8p) translocations, chromosome changes causing WHS are not related either to segmental duplications, or to a parental 4p16 inversion polymorphism.

From these observations, one can infer that many chromosomal changes affecting different chromosomes are neither generated by segmental genomic duplications nor by inversion polymorphisms. Accordingly, detecting such polymorphisms in healthy individuals does not seem to represent a risk factor for meiotic chromosome rearrangements.

On a clinical point of view, WHS cannot be considered a unique disease, due to the great variability of the basic genomic defect, as we demonstrated.

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