Am. J. Hum. Genet. 50:902–913, 1992

Waardenburg Syndrome (WS) Type I Is Caused by Defects at Multiple Loci, One of Which Is Near ALPP on Chromosome 2: First Report of the WS Consortium

Lindsay A. Farrer,* Kenneth M. Grundfast,‡ Jean Amos,† Kathleen S. Arnos,§ James H. Asher, Jr.,‖ Peter Beighton,# Scott R. Diehl,**,†† Jörgen Fex,‡ Carole Foy,‡‡ Thomas B. Friedman,‖ Jacquie Greenberg,# Christopher Hoth,† Mary Marazita,** Aubrey Milunsky,† Robert Morell,‖ Walter Nance,** Valerie Newton,§§ Rajkumar Ramesar,# Theresa B. San Agustin,‡ James Skare,† Cathy A. Stevens,*,[1] Ronald G. Wagner, Jr.,‡ Edward R. Wilcox,‡ Ingrid Winship,# and Andrew P. Read‡‡

*Department of Neurology and School of Public Health and †Center for Human Genetics, Boston University School of Medicine, Boston; ‡Laboratory of Molecular Otology, National Institute on Deafness and Other Communication Disorders, National Institutes of Health, Bethesda, MD; §Gallaudet University, Washington, DC; ‖Department of Zoology and Genetics Graduate Program, Michigan State University, East Lansing; #Department of Human Genetics, University of Cape Town, Cape Town; Departments of **Human Genetics and ††Psychiatry, Medical College of Virginia, Richmond; and ‡‡Department of Medical Genetics and §§Center for Audiology, University of Manchester, Manchester, United Kingdom

Summary

Previous studies have localized the gene for Waardenburg syndrome (WS) type I to the distal portion of chromosome 2q, near the *ALPP* locus. We pooled linkage data obtained from 41 WS type I and 3 WS type II families which were typed for six polymorphic loci on chromosome 2q in order to refine the location of the WS locus (*WS1*) and evaluate the extent of genetic heterogeneity. In the course of this work, we developed diagnostic criteria for genetic and phenotypic studies. Our findings, based on two-locus and multilocus analysis using a linkage map established from reference pedigrees, suggest that there are two or more mutations causing WS, one of which (i.e., *WS1*) is located on chromosome 2q, between the *ALPP* and *FN1* loci, at distances of 7.8 cM and 11.2 cM for each marker, respectively. The results also indicate that WS1 is responsible for the illness in approximately 45% of all families in this sample. However, the odds favoring this position over a location between *ALPP* and *SAG* are only 2:1 when alternate assumptions about the proportion of linked families are considered. We conclude that a more saturated map of this region of chromosome 2q, including highly polymorphic markers, will be needed to accurately distinguish linked families and, ultimately, isolate the mutant gene.

Introduction

Waardenburg syndrome (WS) is an autosomal dominant disorder which has been subcategorized into WS type I (MIM 19350) and WS type II (MIM 19351) on the basis of the presence or absence of dystopia canthorium (lateral displacement of the inner canthi). Manifestations of WS which vary within and between families include sensorineural deafness and pigmentary disturbance of the skin (congenital leukoderma), iris (heterochromia irides and hypoisochromia irides), and hair (white forelock and premature graying), together with minor abnormalities of the periorbital and periodontal structures. In rare instances the disorder may be nonpenetrant. Dystopia canthorum is the most consistent feature of WS type I, occurring in 98% of cases (Waardenburg 1951; Hageman and Dellman 1977). While 50% of WS type II patients have hearing

Received October 15, 1991; revision received January 13, 1992.

Address for correspondence and reprints: Lindsay A. Farrer, Ph.D., Department of Neurology, Boston University School of Medicine, 80 East Concord Street, Boston, MA 02118.

1. Present address: Department of Pediatrics, University of Tennessee, P. C. Thompson Children's Hospital, Chattanooga, TN.

0002-9297/92/5005-0004$02.00

impairment (Arias 1971) and while the frequency may be 20 times that in WS type I (Arias and Mota 1978), WS is responsible for about 2% of congenital deafness (Waardenburg 1951; Partington 1964; Sellars and Beighton 1983). The population frequency of WS is approximately 1/42,000, with an incidence of 1/270,000 births (Waardenburg 1951).

The recent finding of a de novo paracentric inversion in a child with classical WS type I features (Ishikiriyama et al. 1989) provided a clue to a potential chromosomal location of a WS gene. The breakpoints of the inversion are at 2q35 and 2q37.3. Foy et al. (1990) demonstrated linkage of the WS locus (*WS1*) to *ALPP* (alkaline phosphatase, placental) in five families from the United Kingdom, with a peak lod score ($\hat{Z}$) of 4.76 at a recombination frequency (θ) of .023. Linkage of *WS1* to *ALPP,* with no recombination, was subsequently detected in a single large Michigan kindred (Asher et al. 1991).

In an attempt to clarify the extent of linkage heterogeneity of WS type I and refine the location of the *WS1* gene on chromosome 2q, six groups of investigators pooled linkage information on a large set of WS families. A third objective of this collaborative effort was to develop uniform diagnostic criteria to facilitate gene mapping studies and evaluation of phenotypic variation of WS.

Subjects and Methods

Subjects and Diagnostic Criteria

Forty-four kindreds comprising 546 individuals were analyzed for six DNA marker loci on chromosome 2q. Selected characteristics of each family are listed in table 1. These families, ascertained in the United States, the United Kingdom, and South Africa, represent a variety of ethnic backgrounds. Clinical manifestations of affected members in families 16, 17, 37–39, 41, and 42 have been described elsewhere (Foy et al. 1990; Asher et al. 1991; Winship and Beighton, in press). Lod score (Z) data for *ALPP* in five of the UK families and for all of the marker loci except *SAG* in the large kindred from Michigan were previously published (Foy et al. 1990; Asher et al. 1991).

Because the phenotypes of WSs are variable even within a family and may be nonpenetrant in some individuals, diagnosis is not easily made in all cases. In order to minimize misclassification of subjects, a subgroup of consortium investigators (K.M.G., K.S.A., P.B., A.M., W.N., T.B.S., and C.A.S.) identified major, minor, and rare diagnostic features of WS (listed in the Appendix). We agreed that to be classified as affected, an individual must have either (*a*) at least two of the major criteria or (*b*) one of the major criteria and two of the minor criteria (not including the rare criteria). The association of the rare findings and WS is recognized, but at the present time we believe that they should not be used in establishing a diagnosis.

Because of the importance of dystopia canthorum in making the diagnosis of WS type I, the following measurements were taken: A (inner canthal distance), B (interpupillary distance), and C (outer canthal distance). Two indices were chosen as sensitive measures of the presence of dystopia canthorum (Arias 1971; Arias and Mota 1978). The index $W-W = X + Y + (A/B)-$is used when all three measurements are available, while $X-X = [2A-(0.2119C+3.909)]/C-$is used when the interpupillary distance is missing. The value of Y is given by $Y = [2A-(0.2497B+3.909)]/B$. The constant values presented in the above equations were derived from a discriminant analysis (Cotterman 1951) of the differences between the measures of A, B, and C from normal and WS type I subjects, which produced the following discriminant function: $L = A-0.2497B-0.2119C-3.909$. According to Arias and Mota (1978), no typical WS type I subject was ever observed with a W index below 2.07.

Of the persons typed for RFLPs, 274 were classified as affected, 268 were classified as unaffected, and 4 were designated as uncertain. The WS phenotype in 36 untyped founders was considered as unknown because of insufficient information. Three families (8, 14, and 44) were classified as WS type II on the basis of absent dystopia canthorum in affected members.

DNA Analysis

DNA extraction, restriction-endonuclease digestion, agarose-gel electrophoresis, Southern blotting, and hybridization were carried out using standard techniques (Feinberg and Vogelstein 1983; Feder et al. 1985). The WS family members were typed for alleles described in table 2. These markers were studied because they have been mapped, by either genetic linkage analysis (O'Connell et al. 1989; Pakstis et al., in press) or somatic cell hybrids and in situ hybridization (Martin et al. 1987*b*), to the putative region containing *WS1,* as suggested by cytogenetic analysis of the patient with the inversion at 2q35–q37 (Ishikiriyama et al. 1989).

Table 1

Selected Characteristics of WS Families

Family (ID)	Ethnic Origin	WS Subtype	No. of Generations Typed	No. of Individuals Typed		
				Affected	Unaffected	Total
1 (BU1)	Irish	I	3	9	1	10
2 (BU2)	Italian	I	3	5	4	9
3 (BU3)	Irish and Scottish	I	3	8	6	14
4 (BU4)	Jewish	I	3	3	3	6
5 (BU5)	Western European	I	3	4	3	7
6 (BU6)	Western European	I	3	6	6	12
7 (BU7)	Irish	I	3	4	5	9
8 (BU8)	English	II	3	4	4	9[a]
9 (BU9)	Irish and Dutch	I	3	3	2	5
10 (BU10)	Western European	I	2	4	1	5
11 (BU11)	Polish	I	4	6	7	13
12 (NIH3)	German	I	3	8	4	12
13 (NIH8)	German	I	3	4	1	5
14 (NIH16)	Mixed European	II	3	8	4	12
15 (NIH32)	English	I	3	9	11	20
16 (MSU1)	Caucasian	I	4	11	23	34
17 (UCT1)	Mixed ancestry[b]	I	3	13	15	30[c]
18 (UCT2)	Mixed ancestry	I	3	9	6	16[a]
19 (UCT3)	Mixed ancestry	I	2	3	1	4
20 (UCT4)	Mixed ancestry	I	3	4	7	11
21 (UCT5)	Mixed ancestry	I	3	5	2	7
22 (UCT6)	Mixed ancestry	I	3	4	9	15[c]
23 (UCT27)	Mixed ancestry	I	3	6	6	12
24 (MCV901)	Caucasian	I	3	3	6	9
25 (MCV909)	Caucasian	I	3	33	29	62
26 (MCV913)	Caucasian	I	3	4	4	8
27 (MCV914)	Caucasian	I	3	7	6	13
28 (MCV915)	Caucasian	I	3	5	1	6
29 (MCV916)	Caucasian	I	2	2	2	4
30 (MCV919)	Caucasian	I	2	2	2	4
31 (MCV921)	Caucasian	I	2	6	6	12
32 (MCV922)	Caucasian	I	3	7	6	13
33 (MCV923)	Hispanic	I	4	7	6	13
34 (MCV926)	Caucasian	I	3	3	4	7
35 (MCV927)	Caucasian	I	3	7	3	10
36 (MCV928)	Caucasian	I	2	2	2	4
37 (UM1)	English	I	3	6	14	20
38 (UM2)	English	I	3	6	3	9
39 (UM3)	English	I	3	7	6	13
40 (UM4)	English	I	3	3	3	6
41 (UM5)	English	I	3	5	4	9
42 (UM6)	Welsh	I	3	7	5	12
43 (UM7)	English	I	3	4	4	8
44 (UM8)	English	II	4	8	11	19
Total				274	266	546

[a] Includes one uncertain diagnosis.

[b] South African kindreds derived from several ancestral sources: San (Bushmen), Xhoi (Hottentot), Madagascan, and west African black, western European, and Javanese.

[c] Includes two uncertain diagnoses.

Table 2

Chromosome 2q DNA Polymorphisms Typed in Present Study

Locus Symbol and Probe Name	Restriction Enzyme	Fragment Size(s) (kb)	Published Frequency	Estimated Frequency	Reference
ALPP:					
ALP-1	*Rsa*I	1.6	.38	.38	Martin et al. 1987*a*
		1.8	.62	.62	
CRYG:					
p5G1	*Taq*I	3.5	.68	.68	Meakin et al. 1985
		3.3	.32	.32	
	*Taq*I	2.2	.33	. . .[a]	Meakin et al. 1985
		1.1	.67		
D2S3:					
p5-1-30	*Hin*dIII	9.3	.18	.31	Litt and White 1985
		4.6	.82	.69	
p5-1-32	*Hin*dIII	14.5	.20	.18	Litt and White 1985
		12.5	.80	.82	
p5-2-96	*Taq*I	2.75	.21	.22	Litt and White 1985
		2.6	.79	.78	
p5-1-25	*Pst*I	VNTR	PIC = .81		Litt and White 1985
D2S55:					
pEKZ105 ...	*Rsa*I	3.0	.46	.47	Kumin-Wolff et al. 1987
		2.6	.54	.53	
FN1:					
pFH154	*Msp*I	0.35	.75	. . .[a]	Gardella et al. 1988
		2.7	.25		
	*Hae*III	1.5	.39	.40[b]	Colombi et al. 1987
		2.0	.61	.60	
pFH6	*Hin*dIII	7.1	.13	. . .[a]	Colombi et al. 1988
		3.7 and 3.4	.87		
	*Hae*III	1.5	.39	.40[b]	Colombi et al. 1987
		2.0	.61	.60	
SAG:					
S-Ag	*Rsa*I	4.2	.73	.76	Ringens et al. 1990
		3.9	.27	.24	

[a] Frequencies were not estimated because polymorphism was scored in small minority of families.
[b] Estimated frequencies exclude South African kindreds (pedigrees 17–23 in table 1).

Statistical Analysis

Genotypic data were entered into a computerized data base. Data were checked for errors, manipulated for haplotyping, and formatted for linkage analyses with the assistance of the IMAGENARI software (Farrer 1986). Loci detected by multiple probe/enzyme combinations were haplotyped by hand. The scheme of Braverman (1985), which reduces the number of parental alleles to three, made feasible joint analysis of several highly polymorphic loci. Linkage analysis was performed with the programs LIPED version 3 (Ott 1974), LINKMAP version 4.9 from the LINKAGE package (Lathrop et al. 1984), and MENDEL (Lange and Boehnke 1983). An autosomal dominant mode of inheritance, 93% penetrance for the WS trait in both sexes (Badner and Chakravarti 1990), and a WS gene frequency of .001 were assumed. The actual penetrance of WS in families without the occurrence of Hirshsprung disease may be closer to 100%, and the gene frequency may be much smaller than .001; however, the more conservative values were used instead, to allow for mildly affected individuals who may not have been detected clinically. Allele frequencies for the pFH154/*Hae*III polymorphism in the South African kindreds were set at .5 on the basis of evaluation of 17 unrelated individuals from that population. Maximum likelihood estimates of the marker-allele frequencies were also obtained from our WS consortium data set by using the method of Boehnke (1991), which corrects for the familial nature of the

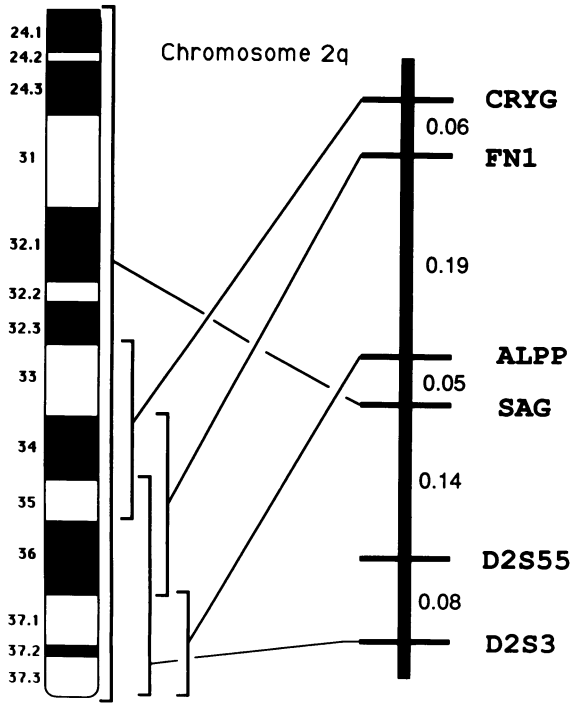

Figure 1 Genetic linkage map of chromosome 2q, comprising the marker loci in table 2. Order of and distances between all loci except for FN1 were derived from multilocus analyses in non-CEPH reference families (Pakstis et al., in press) and are in agreement with the genetic linkage map of O'Connell et al. (1989), which has three loci (*CRYG, D2S3,* and *D2S55*) in common. *FN1* was assigned to the given location on the basis of multilocus analysis in the WS families, which assumed a fixed map distance of 25 cM between *CRYG* and *ALPP* (Pakstis et al., in press). Although the support for this order (multipoint $Z = 3.1$) is only two times greater than the support for the order with *CRYG* and *FN1* inverted, this placement is consistent with the maximum likelihood estimates of recombination for *FN1* vs. *CRYG* ($\hat{\theta} = .07$, $Z = 2.08$) and *ALPP* ($\hat{\theta} = .21$, $Z = 2.11$) in the WS families. The locus order is also consistent with known physical mapping assignments indicated by brackets facing the idiogram on the left.

data. As can be seen in table 2, the estimates in our data are consistent with published allele frequencies.

Multilocus linkage analysis was performed to discriminate among seven possible locations in the linkage map of these markers, shown in figure 1. Overlapping five-locus (WS1 plus four marker loci) analyses were carried out in a manner previously described by Bowcock et al. (1987), which allowed both comparison of results across all intervals and efficient computation.

The predivided sample test of Morton (1956) was used to test for heterogeneity in linkage between WS type I families and WS type II families. Linkage heterogeneity among all families and within WS type I was assessed using Smith's admixture test (Smith 1963) and the B test (Risch 1988), implemented in the HOMOG and BTEST programs, respectively (Ott 1985; Risch 1988). For the admixture test, comparisons of the null hypothesis of no linkage (H_0), the alternate hypothesis of linkage homogeneity (H_1; proportion of linked families [α] = 1), and the hypothesis of linkage with heterogeneity (H_2; $\alpha < 1$), for each of the six markers and for all of the markers jointly considered as a linkage group, were carried out as likelihood-ratio tests with P values calculated from the asymptotic χ^2 distribution of the likelihood-ratio statistic with 1 or 2 df. Families having fewer than two informative meioses were excluded from the B test, for which df are determined by the number of families.

The standard implementation of the admixture test (Smith 1963; Ott 1983) identifies the maximum likelihood estimate of both α for a given chromosomal region and the specific map location of the disease gene in the linked subset of families. However, in addition to identifying the maximum likelihood estimates of these two parameters, it is also of interest to consider their confidence intervals. In order to help visualize this two-dimensional likelihood surface, computer programs have been developed to plot the lod score (Z) for a disease locus, across an interval of linked marker loci, for various levels of the heterogeneity hypothesis (Su 1991; S. R. Diehl and C. MacLean, unpublished data). This is done simply by applying the admixture transformation to each family's data, where L_i is the likelihood ratio of the family's data in a comparison of support of free recombination (i.e., the antilog 10 of the family's Z): $Z(\alpha, \text{location}) = \log [(\alpha)(L_i) + (1 - \alpha)]$. The sum of the admixture-transformed Z values across all families in the study represents the Z for the specific hypothesis for α at a specific map location. By calculating this statistic for a range of α values and map locations and plotting the results, the relative support for alternative map locations for different hypotheses about α for these locations can be visualized directly.

Results

Pairwise Comparisons

Table 3 shows the Z values for various θ values, including the maximum likelihood estimate of recombination ($\hat{\theta}$) between *WS1* and the chromosome 2 loci. On the assumption of homogeneity, significant evidence for loose linkage was obtained for *ALPP,* at $\hat{\theta}$

Table 3

Results of Pairwise Analyses between *WS1* and Marker Loci in 44 WS Kindreds

	Z AT θ OF									
LOCUS	.00	.001	.01	.05	.10	.20	.30	.40	$\hat{\Theta}$	$\hat{Z}$
ALPP	−21.12	−18.14	−8.32	1.69	5.23	6.12	4.17	1.59	.17	6.31
CRYG	−16.13	−12.43	−8.86	−4.92	−2.91	−1.10	−.35	−.07	.50	0
D2S3	−45.52	−41.79	−31.06	−16.02	−8.30	−1.91	.07	.33	.37	.35
D2S55	−43.87	−37.18	−26.24	−13.28	−6.88	−1.74	−.10	.14	.38	.15
FN1	−22.28	−20.13	−13.28	−4.94	−1.41	.85	.94	.36	.25	1.04
SAG	−16.76	−12.24	−8.82	−5.12	−3.21	−1.34	−.51	−.16	.50	0

= .17. The 95% confidence limits for $\hat{\theta}$ (Conneally et al. 1985) are .10 and .25. Corroborating evidence for linkage of *WS1* to this region of chromosome 2 is provided by *FN1* ($\hat{Z}$ = 1.0). *WS1* is excluded at a distance of approximately 15–20 map units from *D2S3, D2S55,* and SAG. More than 10 map units are excluded around *CRYG.* No significant sex differences in recombination fractions between *WS1* and any of the marker loci were observed.

Heterogeneity Testing

On the basis of the *ALPP* results, there was significant evidence for heterogeneity between WS type I families and WS type II families (χ^2_1 = 7.51, P = .006). In addition, there was significant evidence against linkage in the WS type II families ($\hat{Z} \leqslant -2$ at $\theta \leqslant 0.1$). However, heterogeneity between WS types I and II could not be evaluated for any of the other markers, because none of these loci were significantly linked in either subset of families. Evaluation of the pairwise Z values by using the admixture test showed that there is no evidence for linkage to *CRYG, D2S3, D2S55,* and *SAG,* in any subset of families (table 4). However, the hypothesis of linkage homogeneity (i.e., H_1) between *WS1* and *ALPP* is strongly rejected in favor of a model in which an estimated 55% of families are closely linked (θ = .05) and the other 45% are unlinked (H_2). The conclusion of heterogeneity for WS was unaltered by removing from the analysis the three WS type II families. These results suggest that WS type I is a genetically heterogeneous disorder. Analysis of these data by the B test, which is less sensitive to both family size and the true recombination frequency (Risch 1988), supports genetic heterogeneity both in (*a*) all families in the data set (test statistic = 6.34, P = .006) and (*b*) WS type I families alone (test statistic = 2.76, P = .048).

Further support for the existence of a chromosome 2–linked locus for WS comes from findings with *FN1.* Whereas consideration of the entire sample of families does not conclusively show linkage to *FN1* (table 3), there is significant evidence for both heterogeneity among Z values and, in approximately 30% of families, linkage, without recombination, to this locus (table 4; B test results: all families—test statistic = 9.29, P = .001; WS type I families only—test statistic = 9.17, P = .001). Most of the families showing linkage to *FN1* also appear linked to *ALPP.* An exception is family 17, which is apparently unlinked to *ALPP* (Z = −2.2 at θ = .04) but is strongly linked to *FN1* (Z = 3.2 at θ = 0) and possibly linked to *D2S55* (Z = 0.98 at θ = 0). Careful inspection of the data for this family revealed three obligate and as many as several inferred crossovers with *ALPP,* under the most parsimonious linkage phase. Two of the three meioses in question were uninformative for *FN1* and were only partially informative for *D2S55.* On the basis of pairwise analysis, it is inferred that in this family there is recombination between *WS1* and *ALPP.* Evaluation of heterogeneity among location scores from multilocus analysis showed that the proportion of families linked to this region of chromosome 2 (45%) is less than estimated on the basis of the Z data from *ALPP* alone, indicating that in some families positive linkage results with *ALPP* may be spurious. It is noteworthy that, when data on *ALPP* and *FN1* are jointly considered, the conditional probability of linkage for family 17 is 98.9%.

Multilocus Mapping

Multilocus linkage analyses were carried out using the genetic linkage map, to determine the precise location of WS among the group of markers. Under the assumption of linkage homogeneity for WS, the *WS1* locus is excluded within 13 cM proximal to *CRYG,* within 18 cM distal to *D2S3,* and at every location

Table 4

Tests for Genetic Heterogeneity in WS

Marker and Sample (no. of informative families)	Parameter Estimate[a]			Tests of Hypotheses			
				H_2 vs H_1		H_2 vs H_0	
	Θ_1	Θ_2	α	χ_1^2	P	χ_2^2	P
ALPP:							
WS types I and II (28)	.20	.05	.55	6.91	.004	35.15	<.0001
WS type I only (24)	.10	.05	.65	3.67	.028	39.08	<.0001
CRYG:							
WS types I and II (9)	.5	.5	—	.00	.5	.00	.5
WS type I only (8)	.5	.5	—	.00	.5	.00	.5
D2S3:							
WS types I and II (22)	.40	.10	.25	1.02	.16	2.54	.14
WS type I only (22)	.40	.10	.25	1.17	.14	2.73	.13
D2S55:							
WS types I and II (24)	.40	.30	.40	.60	.22	1.20	.22
WS type I only (22)	.40	.30	.50	.84	.18	1.62	.22
FN1:							
WS types I and II (22)	.30	.00	.30	10.22	.0007	14.60	.0003
WS type I only (21)	.30	.00	.25	9.76	.0009	13.40	.0006
SAG:							
WS types I and II (11)	.5	.5	—	.00	.5	.00	.5
WS type I only (10)	.5	.5	—	.00	.5	.00	.5
Multilocus:[b]							
WS types I and II (34)	.095	.114	.45	36.18	<.0001	38.31	<.0001
WS type I only (31)	.095	.114	.50	29.04	<.0001	39.63	<.0001

[a] $\Theta_1 = \theta$ when linkage homogeneity is assumed; and $\Theta_2 = \theta$ when heterogeneity is assumed for linked families.

[b] Evaluation of location scores from multilocus analysis when a single *WS* locus within a cluster of linked markers is assumed; values for Θ_1 and Θ_2 are the most likely location for *WS1* on the linkage map (see fig. 1) when FN1 is assumed to be at position 0 and when ALPP is assumed to be at position .19.

between *CRYG* and *D2S3* (a region encompassing approximately 52 cM), with the exception of an 8-cM region in the *FN1–ALPP* interval, in which the multilocus Z reaches a maximum of 0 (fig. 2). Nevertheless, there is no evidence for linkage in this region of chromosome 2 or within 50 cM on either side of the linkage group. The finding was not unexpected, in light of the evidence favoring heterogeneity and linkage in a minority of families. In an attempt to clarify the locus-ordering problem, the statistical support at various locations on the genetic map was recalculated under different assumed values of α. The peak location score was obtained under the hypothesis of $\alpha = .45$ (i.e., the maximum likelihood estimate of α), suggesting that *WS1* is most likely positioned 11.4 cM distal to *FN1* and 7.6 cM proximal to *ALPP* (see table 4 and fig. 2). All other locus orders were significantly ruled out, except for the order *ALPP–WS1–SAG*, which was only six times less likely. However, when $\alpha = .25$, this latter order is only two times less likely than the most favored order.

Discussion

Two independent studies have convincingly demonstrated the existence of a gene for WS type I on chromosome 2q, near the ALPP locus (Foy et al. 1990; Asher et al. 1991). Of the six reported families, none appeared to be excluded from close linkage to *ALPP*. In the mouse, mutations at several different loci correspond to phenotypes possibly analogous to WS (Asher and Friedman 1990), suggesting that there could be two or more unlinked mutations causing WS in man. However, pooling linkage data from a large number of families was required to formally test this hypothesis. The pooled results are consistent with genetic heterogeneity for WS type I. In addition, at least two of the three WS type II families appear to be unlinked to this region of chromosome 2.

The information in this sample of 44 kindreds indicates that a mutant gene adjacent to the *ALPP* locus on chromosome 2q is responsible for WS in approximately 45% of families. The results of our analyses

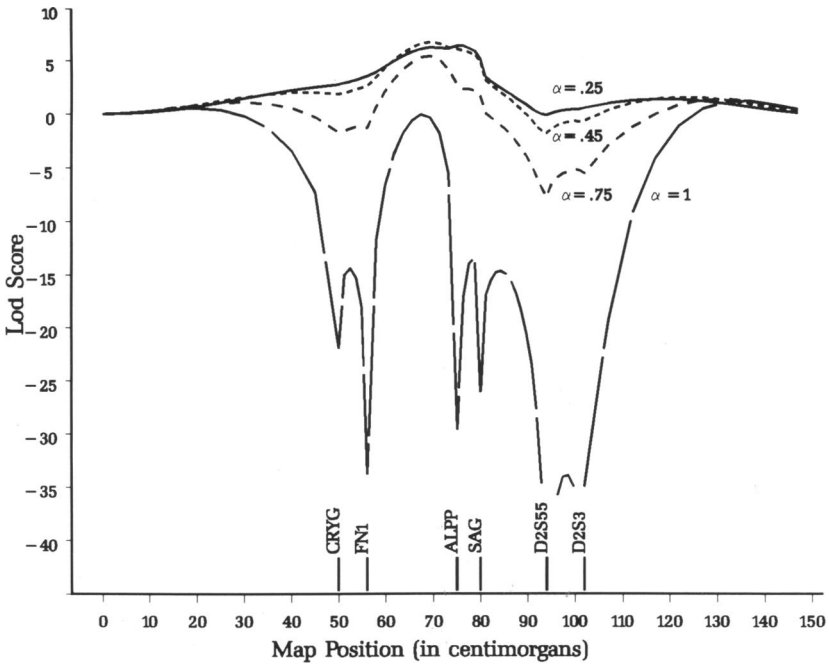

Figure 2 Support for position of WS1 with respect to chromosome 2 marker loci, according to the assumption of α. The position of *CRYG* was arbitrarily set at 50.0, and the positions of the other loci were fixed according to the genetic map in fig. 1. Curves are presented for α = 1 (i.e., all families included), α = .45 (the maximum likelihood estimate for α), and two other representative α values. A $\hat{Z}$ of 6.79 was obtained at a location between *FN1* and *ALPP* when α = .45.

suggest that the true θ value between *WS1* and *ALPP* is .076. The present study further suggests that *WS1* is only two times more likely to be located in the interval between *ALPP* and *FN1* than in the adjacent interval, between *ALPP* and *SAG*. Unfortunately, the *SAG* polymorphism was not very informative in this data set. Therefore, distinction among these two orders remains equivocal. Other orders are excluded by odds of more than 100:1, regardless of the assumed α value. Note that these odds are predicated on the preliminary linkage map established for this region of chromosome 2q. Our results should be interpreted cautiously until a better-supported and more saturated map of this region of chromosome 2q is available. Accuracy of the precise location of *WS1* and distance estimates should improve with successive refinements and with addition of new highly polymorphic loci to the map.

The large confidence interval for α (.15–.7) and, consequently, the large confidence interval for θ between *WS1* and *ALPP* (.02–.15) caution against immediate use of this tantalizing finding to attempt cloning of the gene, until a more definitive estimate of θ is obtained. This could be accomplished by studying the *ALPP*, *FN1*, and *SAG* polymorphism in additional large WS kindreds. However, a more effective method for discerning linked from unlinked families would be to identify a set of highly polymorphic flanking markers approximately 1–3 cM apart that define the smallest cosegregating region for *WS1*. Application of the interval mapping method (Lander and Botstein 1986) would then accurately detect unlinked families as double crossovers within the smallest cosegregating region, which, in a region this size, normally occur with a frequency of less than 0.1%. A narrowly defined cosegregating region would also facilitate methods for cloning the gene, because it delineates the boundaries and the approximate molecular distance of the region that must be screened.

The suggestion for genetic heterogeneity in WS has come from sources other than linkage studies. Subjects have been divided, on the basis of particular phenotypic constellations, into four subgroups including the common WS types, I and II. Patients having the WS type I phenotype in association with upper-limb abnormalities may be classified as WS type III, or Klein-Waardenburg syndrome (MIM 14882; Goodman et al. 1982; Klein 1983). WS type IV, or Waardenburg-Shah syndrome (MIM 27758), is a rare autosomal recessive disorder that is distinguished by the association of megacolon (Hirshsprung disease) with several

characteristics of type I (Omenn and McKusick 1979; Kelley and Zackai 1981; Shah et al. 1981; Ambani 1983; Meire et al. 1987). However, this condition bears little resemblance to classical WS (McKusick 1988) and does not require further consideration in this context. None of the individuals studied here has Hirshsprung disease or upper-limb abnormalities.

Comparisons of syntenic relationships of murine mutants exhibiting pleiotropic effects similar to WS with homologous regions on the human genetic map also predict multiple candidate genes for WS (Asher and Friedman 1990). For example, mice heterozygous for *Sp* (Splotch), *Mi^{or}* (Micropthalmia Oak Ridge), and *Ph* (Patch) are phenotypically similar to WS. Synteny of the mouse *Sp* mutation predicts a human homologue on 2q, near *FN1: COL6A3, ALPP,* and *D2S3*. The synteny of *Mi^{or}* predicts a human homologue near *RAF1* on 3p or near *RHO* on 3q. Similarly, synteny of *Ph* predicts a human homologue on 4q, near the *KIT* proto-oncogene. Coincidentally, a Gly→Arg substitution at codon 664 within *KIT* has recently been identified as a cause of piebaldism (Giebel and Spritz 1991), a dominant disorder which is characterized by congenital hypopigmented patches of skin and hair and which may share a common embryologic origin with WS.

The conclusion of linkage heterogeneity for WS, a complex phenotype with variable expression and reduced penetrance, has the obvious caveat of diagnostic accuracy. In many families, as few as one or two incorrect diagnoses could lead to the false conclusion of nonlinkage. To address this problem, we have developed a scheme for standardization of diagnosis and improvement of diagnostic accuracy. Nonetheless, the possibility of phenotypic misclassification still exists. A more precise method for evaluating the genotype for a complex discrete trait such as WS is the use of a quantitative biological marker. Although efficiency of detecting linkage between a closely linked marker and a quantitative trait may be reduced by as much as 50% if the genotypic means are separated by as few as 3 SD (Ott 1985), the increased information afforded by a phenotype defined with quantitative biological measurements can lead to a substantial improvement in the *Z* value, by contributing to the identification of heterozygotes (Lalouel et al. 1985). The quantitative measure of dystopia canthorum used in the analysis by Asher et al. (1991) resulted in a 20% higher *Z* value than could have been obtained by defining WS on the basis of affection status. A robust measurement of dystopia canthorum in studies involving multiple families must take into account ethnic influences on the anatomical relationships of facial features (Omotade 1990). Our group is currently collecting a variety of ocular measurement data in order to identify a quantitative characteristic that best discriminates genotypes underlying the WS phenotype.

There are no apparent clinical differences among linked and unlinked WS type I families; however, this has not been formally investigated. In the absence of knowledge of the biochemical defect, identification of a biological marker for patients with a mutation in *WS1* (on chromosome 2q) may be difficult because of both high phenotypic variability within families and the probable existence, within WS type I, of multiple mutations which may be associated with unique patterns of expression. It remains to be seen whether the current nosology of WS has a genetic basis.

Note added in proof. — After this work was submitted for publication, two groups in our consortium identified two different exonic mutations within the HuP2 gene at 2q35-q37 that cause WS (Baldwin et al. 1992; Tassabehji et al. 1992). HuP2 is the human homologue of the murine Pax-3 paired domain gene that causes the splotch phenotype (Epstein et al. 1991).

Acknowledgments

We wish to recognize the director, Dr. James Snow, and staff of the National Institute on Deafness and Other Communication Disorders for their support of the Waardenburg Syndrome Consortium effort. Dr. Andrew Pakstis provided prepublished information about his genetic linkage map of chromosome 2. We are grateful to the following individuals for their contributions to this research: Michelle Babaya, Joan Bodurtha, Bettie Duke, Jonathan Gal, Marlene Goedhart, Jamie Israel, Dan Kiely, Barbara Landa, Dr. Lynn Ploughman, Brenda Rawlings, Elizabeth Remington, Amy Terstereip, and Laura Wright. The manuscript was expertly prepared by Jemma Williams. Drs. Robert Martell, Diana Curtis, and Karen Young contributed to the South Africa project. We also thank the following individuals for providing us with probes: Drs. John Trowsdale and Ian Mockridge (ALP-1); Dr. Mon-Li Chu (p5G1), Dr. Francisco Baralle (pFH6 and pFH154), and Dr. Toshimichi Shinohara (S-Ag). Probes for *D2S3, D2S55,* and *FN1* were obtained from the ATCC. This work was supported in part by NIH grant DC00038 (to S.R.D., Principal Investigator), by NIH contract 263-MD-117512 (to L.A.F., Principal Investigator), and by grants from the following: the Grants-In-Aid program of Virginia Commonwealth University (support to S.R.D., Principal Investigator), the South African Medical Research Council, the Mauerberger Foundation, the Harry

Crossley Fund, the University of Cape Town Staff Research Fund, and the Deafness Research Foundation in New York. L.A.F. is a Fellow of the Alfred P. Sloan Foundation.

Appendix

Diagnostic Criteria for WS Type I

Major:

1. Sensorineural deficit hearing threshold greater than 25 dB for at least two frequencies between 250 and 4,000 Hz, without evidence of middle-ear effusion and without evidence to suggest that presbyacusis or other factors account for the elevated hearing threshold
2. Iris pigmentary abnormality:
   a. Two eyes of different color
   b. Iris bicolor/segmental heterochromia—an eye with two different colors. The pattern of the different color within the iris usually occurs in a radial segmental distribution and is clearly demarcated.
   c. Characteristic brilliant blue (sapphire) iris (alternatively described as "Waardenburg blue eye," sky-blue eyes, or hypopigmented iris). Abnormality of the stroma can be confirmed with slit lamp.
3. Hair hypopigmentation:
   a. White forelock: can be present at birth and then disappear later in life, with reappearance in teens or adulthood, or may appear for the first time at any age
   b. Body hair: white hairs within eyebrow, eyelashes, or at other sites on the body
4. Dystopia canthorum: Lateral displacement of inner canthi, with a reduction of visible sclera medially. W index (Arias and Mota 1978) equal to or greater than 2.07. If interpupillary measurements are unavailable, X can be used.
5. First-degree relative previously diagnosed with WS

Minor:

1. Congenital leukoderma—several areas of hypopigmented skin
2. Synophrys or medical eyebrow flare (a flaring or fanning out of the eyebrow hairs medially, toward the nasal bridge)
3. Broad high nasal root
   a. Evident on full-face view
   b. May or may not occur along with absence of nasofrontal depression evident on profile view (high nasal root); there are ethnic variances in facial morphology, and these could influence the degree to which the broad and/or high nasal root would be a dysmorphic feature—and, in turn, the degree to which such findings might be of value in differentiating affected from nonaffected individuals.
4. Hypoplasia of alae nasi—hypoplastic lower lateral cartilages, usually resulting in narrow lower third of the nose
5. Premature graying of the hair—predominance of white scalp hairs before age 30 years; white hairs appearing in the midline rather than at the temples is suggestive of WS.

Rare:

1. Hirschsprung disease
2. Sprengel anomaly
3. Spina bifida
4. Cleft lip and/or palate
5. Limb defects
6. Congenital heart abnormalities
7. Abnormalities of vestibular function
8. Broad square jaw
9. Low anterior hair line

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