MUTATION IN BRIEF

Werner Syndrome and Mutations of the *WRN* and *LMNA* Genes in France

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Werner syndrome (WS) is a pleiotropic disease of premature aging involving short stature, tight, atrophied, and/or ulcerated skin; a characteristic 'birdlike' facies and high, squeaky or hoarse voice; premature greying and thinning of the hair; and early onset cataracts. Additional common symptoms include diabetes mellitus, hypogonadism, osteoporosis, osteosclerosis of the digits, soft tissue calcification, premature atherosclerosis, rare or multiple neoplasms, malformed teeth, and flat feet. Diagnosis can be difficult due to the variable presentation and rarity of the disorder. Transmission is usually autosomal recessive. The WS gene, *WRN*, is member of the RecQ DNA helicase family. Biallelic mutations of *WRN* are responsible for most patients. Although heterozygous missense mutations in the *LMNA* gene have been observed in severely affected WS patients, this only accounts for a small fraction of non-*WRN* patients. Eighteen WS cases were referred to us for molecular analysis. Eleven had definite and three had probable WS according to the University of Washington Registry clinical criteria. All exons of the *WRN* gene and their splice junctions were sequenced. Of the fourteen definite or probable cases, 11 had one or more WRN mutation. Thirteen different mutations were found, and ten of these were previously undescribed. There were few phenotypic differences between patients with *WRN* mutation(s) and those who met clinical criteria though lacking WRN mutations. However, patients with mutations tended to have more symptoms overall, and mutations were not observed in the two cases with cardiomyopathy. © 2006 Wiley-Liss, Inc.

KEY WORDS: Werner syndrome; *WRN*; *LMNA*

INTRODUCTION

Werner Syndrome (MIM# 277700) is a pleiotropic disease of premature aging involving short stature, tight, atrophied, and/or ulcerated skin, a characteristic 'birdlike' facies and high, squeaky or hoarse voice, premature greying and thinning of the hair and early onset cataracts. Additional common symptoms include diabetes mellitus, hypogonadism, osteoporosis, osteosclerosis of the digits, soft tissue calcification, premature atherosclerosis, rare or multiple neoplasms, malformed teeth, and flat feet (Epstein et al., 1996; http://www.pathology.washington.edu/research/werner/ws_wrn.html).

Most patients carry biallelic mutations of the Werner gene, *WRN* (MIM# 604611; also known as RECQL2 or

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RECQ3). WRN is a member of the RecQ DNA helicase family one of whose main functions is in the surveillance of newly synthesized daughter-strands of DNA for incorporation errors, and their repair (Ozgenc and Loeb, 2005). Only recessive, truncating mutations have thus far been associated with the syndrome, suggesting that complete loss of WRN function is necessary to develop symptoms (Oshima et al., 1996; Moser et al., 1999). One hypothesis to explain the symptoms of WS is that in the absense of this helicase, DNA replication is abnormal, leading to cell death and increased tissue turnover.

In about 20 % of cases conforming at least possibly or probably to WS according to the International Registry criteria, no mutation in the *WRN* gene is detected, indicating genetic heterogeneity for this disease (Moser et al., 1999; Chen et al 2003; see also the International Registry of Werner Syndrome). Four of 26 such patients (15 %) were shown to carry dominant missense mutations of the *LMNA* gene; these patients tended to be diagnosed earlier and have more rapid evolution of symptoms (Chen et al, 2003).

Most work on WS has been undertaken in the United States, and also in Japan, where there is an exceptionally high incidence due to founder *WRN* alleles. Less is known about WS in european populations. Since 2001, we have been the reference laboratory for molecular analysis of the *WRN* gene in France. We have used PCR and sequencing to screen for mutations in the *WRN* and *LMNA* genes in 18 suspected WS cases, and correlated these results with phenotypic information.

MATERIALS AND METHODS

Eighteen suspected WS cases were referred to our Center from 1993 to 2005. Patients were referred to our laboratory spontaneously by their own physicians, usually a generalist seeking a molecular confirmation or infirmation of the clinical diagnosis of WS. Most physicians contacted us via the Orphanet online genetic services database (http://www.orpha.net/). Fourteen patients were ethnically French, two were of north African origin (1387-1 and 1199-1), one Indian (1239-1), and one Portugese (1580-1).

For patients referred after 2001, a questionnaire on the symptoms of WS was filled out at the time of referral and informed consent. The form included information on age, height, weight, facial aspect, characteristic voice, dysmorphology, grey/thin hair, baldness, atrophied/thin/wrinkled/ulcerated skin, deafness, cataracts, diabetes/hyperglycemia, osteoporosis of the limbs or trunk, arteriosclerosis, hypertension, hypogonadism, menopause, cancer, Alzheimer's disease, calcification of soft tissues, thin extremities of the limbs, flat feet, irregular dentition, karyotype (if known), and hypersensitivity to medications and/or radiotherapy, with age of onset and details where appropriate. Also requested was information on the numbers of healthy and affected siblings, children, parents, and other relatives, and whether there was any indication of consanguinity in the family. Blood was drawn on Li-heparin and sent to our laboratory for DNA extraction. A small percentage (15 %) of non-*WRN*, or 'atypical progeria', cases have been found to be heterozygous for dominant missense mutations in the *LMNA* gene (Chen et al., 2003).

DNA was extracted from peripheral blood by standard methods. PCR primers flanking *WRN* and *LMNA* exons and splice sites were designed using Primer Express software (sequences available on request) and synthesized by MWG Biotech (Roissy, France). The entire coding sequence was analysed, including all intron/exon junctions and at least 30 bp of intronic flanking sequence for each exon. Amplification was performed using reagents from Applied Biosystems (Evry, France) in a Primus HT thermocycler (MWG Biotech, Roissy, France). PCR consisted of an initial denaturation of 5 min at 95°, 30 cycles of (95° 20s, 54° 20s, 72° 20s) and 5 min extension at 72°, except for exons 10 and 11, which were amplified in a hot start followed by 5 min at 95°, 5 cycles of (95° 20s, 60° 10s, 72° 20s) and 25 cycles of (95° 20s, 56° 10s, 72° 20s) and a final extension of 5 min at 72°. PCR products verified by agarose gel electrophoresis were purified by filtration (Millipore, Molsheim, France) and sequenced with Big Dye version 3 reagents (Applied Biosystems, Evry, France). After purification over sephadex, sequences were resolved on a 3100 capillary sequencer (Applied Biosystems, Evry, France) and analysed with Seqman alignment software (DNA-Star, Madison, USA). All mutations were verified by sequencing a second PCR product from the same DNA sample, and then on a second sample from the patient. The mutation in patient 284-18 was uncovered using the protein truncation test and subsequent sequencing, and was described earlier (Vidal et al., 1998).

Theoretical splicing efficiency was examined using the following publicly accessible web sites: http://genes.mit.edu/burgelab/maxent/Xmaxentscan_scoreseq.html and http://www.genet.sickkids.on.ca/~ali/splicesitefinder.html and http://www.fruitfly.org/.

The reference sequences used were: *WRN* genomic sequence AF181897.1; *WRN* cDNA sequence NM_000553.2; *LMNA* genomic sequence NT_004668.16; *LMNA* cDNA sequences NM_170707.1 (variant 1), NM_005572.2 (variant 2) and NM_170708.1 (variant 3). All sequence changes are given in reference to the coding sequence, with +1 being the A of the initiating ATG.

RESULTS

The known patient characteristics are presented in Table 1. Eleven of the 18 patients had 'classic' or 'typical' symptoms and met the International Registry Diagnostic criteria suggesting 'definite' or 'probable' WS, which included dermatologic changes usually associated with aging (thin, tight, atrophic and/or ulcerated skin, pigmentary alterations, loss of subcutaneous fat), early development of cataracts, short stature, and prematurely grey and/or thin hair, plus a characteristic 'birdlike' facies. All patients exhibited dermatological changes typical of WS. With the exception of patient 1580-01, all patients reporting had grey hair and short stature and only two lacked cataracts (1199-1 and 1338-1 at 30 and 50 years of age, respectively). Reporting of other signs was incomplete, and may be indicative of the variable penetrance and age-dependence of other pathologies associated with WS. Patients were classified as 'definite' WS with all the cardinal signs (facies, dermatologic changes, cataracts, short stature, prematurely grey/thin hair) and at least one secondary sign; as 'probable' if missing one cardinal sign but with at least three secondary symptoms. Case 1580-1 should be excluded from Werner Syndrome on clinical grounds: this patient was referred to us at the age of 11 years, indicating a more severe progeric syndrome (Hegele, 2003). Two patients (1019-1 and 1199-1) had cardiomyopathy, not usually seen in WS but associated with mutations of *LMNA*.

Amplification and sequencing of the entire *WRN* coding sequence and splice junctions (covering 10.7 kb for 12.3 % of the 87 kb genomic sequence), showed that eight patients carried biallelic truncating *WRN* mutations, while one patient (943-01) carried a truncating mutation plus a missense mutation of a conserved, charged residue in the helicase domain (Table 1). All of these cases presented a definite clinical picture of WS. Two patients presenting definite or probable clinical pictures of WS each carried a single truncating *WRN* mutation and no additional sequence changes other than previously described polymorphisms. Three mutations were described previously: c.3690_3693del4 by Oshima et al (1996), and c.1105C>T and c.2665C>T by Yu et al (1996); the others appear to be new to this study. One mutation, c.1165delA, was observed in three patients, including two homozygotes. As one of the homozygotes, 1239-1, was of Indian origin and the other patients of French extraction, this does not represent a local founder allele.

Patient 1338-1 had clinical findings that were not highly suggestive of WS. However he was studied in light of having a deceased sister who was reported to have had WS. This patient did not exhibit any *WRN* mutation, but was homozygous for the rare allele of a polymorphism in intron 12, c.1433-3C>A (allele A, frequency 5.7 % (our unpublished data on >700 chromosomes)), and heterozygous for other polymorphisms in exons 4, 20 and 26 and introns 10, 21, 24 and 25. In this case the search for a large deletion involving exon 13 would be especially interesting, given the rarity of homozygotes for the intron 12 transversion. Such a hypothetical deletion would involve at least exon 13 and at most exons 11 to 19 (exons 10 and 20 were shown to be present in two copies due to the heterozygosity of polymorphisms).

In addition, 23 polymorphisms were observed (Table 2). Twelve of these were in coding regions, including six non-synonymous changes. Four of the exonic and three of the intronic polymorphisms have not been described previously. We did not determine the allele frequencies of polymorphisms other than c.1433-3C>A described above. In most cases, no family data was available to determine phase, however, inspection of the patient genotypes did not suggest any common haplotypes (data not shown). The positions of the mutations and coding sequence polymorphisms are recapitulated in Figure 1.

No *WRN* mutation was observed in the remaining five cases. All cases with no or only one truncating mutation were studied further for mutations in the *LMNA* gene. No mutations were found.

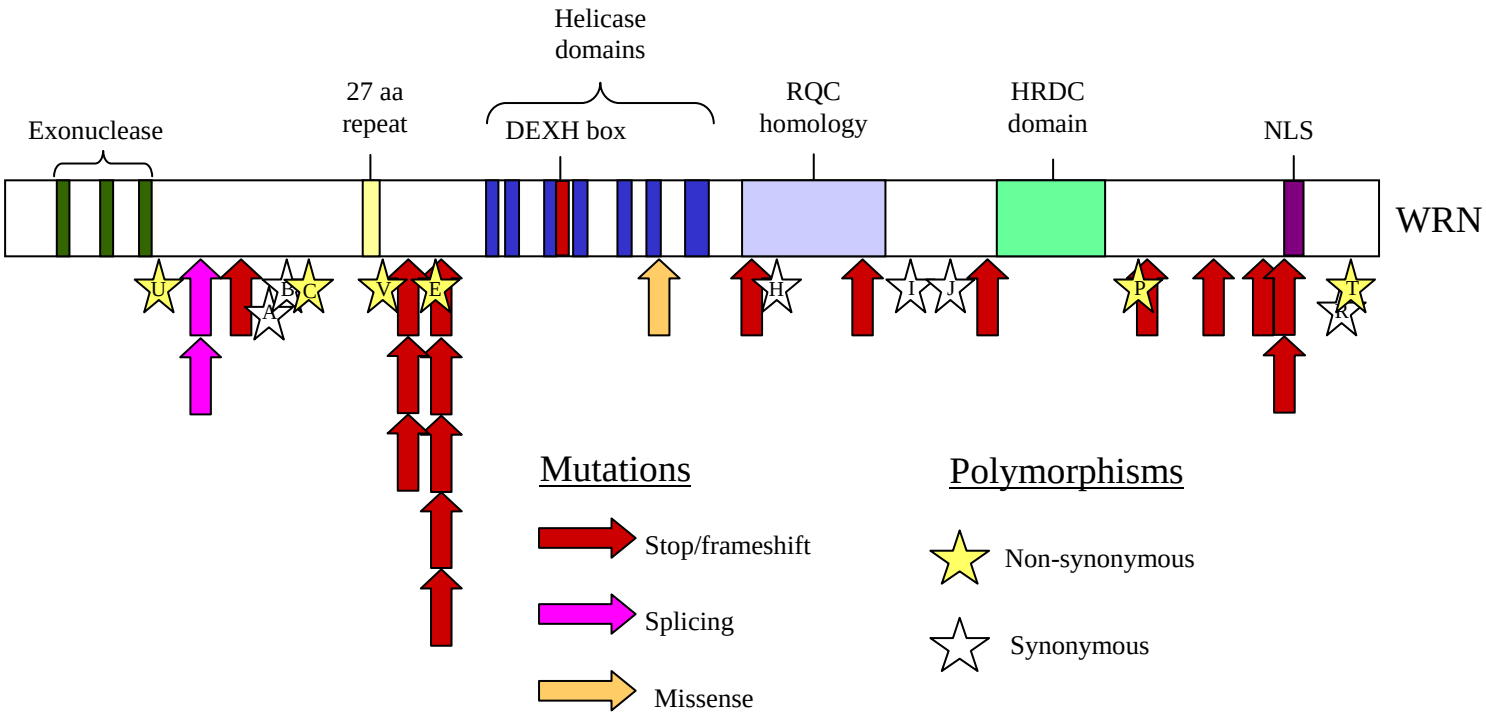

Figure 1. Schematic representation of the WRN protein, from N- to C-terminus. The positions of the mutations described in our patients are indicated with arrows. Synonymous and non-synonymous polymorphisms in the coding sequence are indicated with empty and filled stars, respectively, and coded as in Table 2.

Table 1. Patient characteristics and mutations

Patient	Age[1]	Sex	Facies	WS dermatology[2]	Cataracts	Stature	Aged hair	Family[3]	Secondary symptoms	Diagnosis[4]	Allele 1[5]	Allele 1 effect	Allele 2	Allele 2 effect
284-18	33	F	Yes	Yes	At 27	Short	Yes	Yes	Diabetes, osteoporosis, cancer	Definite	c.1165delA	p.Arg389fs	c.1165delA	p.Arg389fs
1053-1	47	F	Yes	Yes	Yes	Short	Yes	Yes	Diabetes	Definite	c.1105C>T	p.Arg369>X	c.2665C>T	p.Arg889>X
1182-1	42	F	Yes	Yes	Yes	1.57m	Yes	Yes	Possible hypogonadism, soft tissue calcification, flat feet, irregular dentition	Definite	c.2003delAC	p.Asp668fs	c.3690_3693del4	p.Thr1230fs
1239-1	47	M	Yes	Yes	At 45	1.52m	At 15	Yes	Hypogonadism, elevated hyaluronic acid	Definite	c.1165delA	p.Arg389fs	c.1165delA	p.Arg389fs
1387-1	55	F	Yes	Yes	Yes	1.65m	Yes	No	Diabetes, osteoporosis, soft tissue calcification, flat feet, atherosclerosis, irregular dentition	Definite	c.356-2A>C	Skip exon 5	c.356-2A>C	Skip exon 5
1558-1	33	M	Yes	Yes	At 16	1.62m	At 32	Yes	Soft tissue calcification, flat feet, high voice, irregular dentition	Definite	c.474delT	p.Phe158fs	c.1165delA	p.Arg389fs
943-1	48	M	Yes	Yes	Yes	1.67m	Yes	Yes	Osteoporosis, cancer	Definite	c.3244_3245delGT	p.Val1082fs	c.1909A>T	p.Arg637>Trp
1684-1	45	M	Yes	Yes	At 31	1.55m	At 32	No	Soft tissue calcification, flat feet, high voice, cancer	Definite	c.3789C>G	p.Trp1263>X	c.3789C>G	p.Trp1263>X
1801-1	35	M	Yes	Yes	Yes	1.63m	Yes	No	Arteriosclerosis, hypogonadism, high voice, irregular dentition	Definite	c.1105C>T	p.Arg369>X	c.1105C>T	p.Arg369>X
1052-1	53	F	Yes	Yes	n.d.	short	Yes	Yes	Hyperglycemia, atherosclerosis, hearing loss, neuropathy	Probable	c.3496A>T	p.Lys1166>X	None found	
1465-1	46	F	Yes	Yes	Yes	1.52m	Yes	No	Truncal osteoporosis, soft tissue calcification, flat feet, high voice, irregular dentition	Definite	c.2283G>A	p.Trp761>X	None found	
1019-1	48	M	Yes	Yes	n.d.	1.63m	At 17	No	Hypogonadism, cardiomyopathy	Possible				
1016-1	39	F	Yes	Yes	Yes	1.53m	n.d.	Yes	Hyperglycemia, atherosclerosis, osteoporosis	Probable				
1199-1	30	M	Yes	Yes	No	n.d.	Yes	No	Hyperglycemia, hypogonadism, cardiomyopathy	Possible				
1126-1	46	F	Yes	Yes	n.d.	1.40m	At 40	No	Cancer, high voice	Probable				
1407-1	61	M	Yes	Yes	At 40	1.70m	At 30	No	Cancer, irregular dentition, osteopenia	Definite				
1338-1	50	M	Yes	Yes	No	n.d.	n.d.	Yes		Unlikely				
1580-1	11	F	Yes ?	Yes ?	No	1.11m	No	No	Flat feet, high voice, irregular dentition, micrognathism	Excluded				

1, age at referral for molecular analysis; 2, tight, atrophic, and/or ulcerated skin, pigmentary alterations; 3, consanguinity and/or affected sibling; 4, according to the International Registry scoring system; n.d. no data; 5, According to the cDNA coding sequence, with +1 the A of the initiating ATG, cDNA reference sequence NM_000553.2.

Table 2. WRN polymorphisms

Exon/intron	Code (fig. 1)	Nucleotide change[1]	Amino acid change	Described[2]	Patients[3]
Exon 4	U	c.340G>A	p.Val114Ile	Yes	1338-1
Exon 6	A	c.512T>C	p.Cys171 (silent)	Yes	1126-1, 1182-1, 1465-1
Exon 7	B	c.684G>A	p.Asn228 (silent)	No	1182-1
Exon 8	C	c.743G>T	p.Ser248Ile	No	943-1, 1052-1, 1053-1, 1016-1, 1019-1
Intron 8		c.839+14delT		No	1126-1, 1182-1
Exon 9	V	c.970A>G	p.Thr324Ala	Yes	943-1
Exon 9	E	c.1161G>A	p.Met387Ile	Yes	1126-1, 1182-1, 1407-1, 1580-1
Intron 10		c.1350+45T>C		No	1338-1(H), 1407-1, 1558-1, 1580-1
Intron 12		c.1433-3C>A		Yes	1338-1(H)
Intron 14		c.1717+25A>T		Yes	1338-1(H), 1387-1(H), 1407-1
Exon 18	H	c.2040T>C	p.Phe680 (silent)	No	1052-1, 1053-1, 1016-1
Exon 20	I	c.2361T>G	p.Leu787 (silent)	Yes	1016-1, 1126-1, 1199-1(H), 1239-1(H), 1338-1, 1387-1(H), 1407-1, 1558-1(H), 1580-1, 1801-1(H)
Intron 20		c.2274-42C>T		Yes	1387-1(H)
Exon 21	J	c.2520T>C	p.Gly840 (silent)	No	1182-1
Intron 21		c.2448-65G>T		Yes	1338-1, 1387-1(H), 1407-1
Intron 24		c.2826-65delG		Yes	943-1, 1338-1
Intron 25		c.3138+6C>T		Yes	1387-1(H), 1407-1, 1558-1, 1580-1, 1801-1(H)
Intron 25		c.3138+7A>G		Yes	1338-1, 1387-1(H), 1407-1, 1558-1, 1580-1, 1801-1(H)
Exon 26	P	c.3222T>G	p.Phe1074Leu	Yes	1126-1, 1239-1(H), 1338-1, 1387-1(H), 1407-1, 1558-1, 1580-1, 1801-1(H)
Intron 27		c.3309+26C>T		Yes	1558-1, 1580-1
Intron 33		c.3820-97C>T		No	1465-1
Exon 34	R	c.4083C>T	p.Ser1361 (silent)	Yes	1126-1, 1239-1(H), 1558-1, 1580-1
Exon 34	T	c.4099T>C	p.Cys1367Arg	Yes	1199-1(H), 1407-1, 1558-1, 1580-1, 1801-1(H)

All coding polymorphisms not previously described were observed in at least one patient with biallelic *WRN* mutations.
1, According to the cDNA coding sequence, with +1 the A of the initiating ATG, Reference sequences NM_000553.2 (cDNA) and AF181897.1 (genomic); 2, Described in the University of Washington database; 3, (H) homozygous, all other indications are for heterozygosity.

DISCUSSION

Of the 14 cases with definite or probable Werner syndrome, eleven (78.6 %) had at least one mutated *WRN* allele and eight (57 %) had two truncating alleles. Of the twelve different mutations, three have been described previously, while nine are new to this study. Thus far, it is generally agreed that mutations in the *WRN* gene are recessive, and all mutations in the University of Washington database (http://www.pathology.washington.edu/research/werner/ws_wrn.html) truncate the protein with loss of the nuclear localisation signal and are thus predicted to be null alleles, even though for many patients only one mutated allele has been identified (Oshima et al, 1996; Moser et al 1999). It seems likely that the p.Arg637Trp missense mutation in patient 943-1 disrupts the function of the helicase domain and is the first example of a null missense allele in a patient. The amino acid at this position in the mouse *Wrn* homolog and in other RecQ family helicase domains is either lysine (also a positive charge) or glutamic acid (a negative charge). The substitution of bulky, uncharged tryptophan may render the protein non-functional by disrupting its secondary structure or interaction with its substrate. Such an hypothesis may be supported be Kamath-Loeb et al., who demonstrated that a rare polymorphism changing Arginine 834 to Cysteine, also in the helicase domain, is associated with a 40- to 50-fold

reduction in both helicase and exonuclease activity, probably due to the elimination of key hydrogen bonds in the ATP hydrolysis cleft (Kamath-Loeb et al., 2004).

For two other patients, however, we were unable to detect any second mutation in *WRN*. The single, heterozygous *WRN* alleles found in patients 1052-1 and 1465-1 are both truncating mutations similar to those found in patients with biallelic mutations, and the clinical findings are similar as well, giving us no reason to propose that these alleles are dominant. Most likely these patients harbor splicing mutations deep in the introns or rearrangements (e.g., duplications, inversions, large deletions or insertions) not detectable by PCR and sequencing, and thus the second allele appears to be normal by the techniques employed.

In general, *WRN* mutations were found among the patients with the strongest clinical picture of WS, although three patients with definite or probable WS were seemingly without defects in the *WRN* gene. All patients with mutations exhibited the five cardinal symptoms: typical dermatologic changes, early cataracts, short stature and precociously thin and greying hair, with the exception of patient 1052-1 for whom there was no data on the presence of cataracts. These were the only symptoms present in all the patients with mutations. While some of the patients without detectable mutations also fulfilled these criterea, two others did not have cataracts (1199-1 and 1338-1 at 30 and 50 years of age, respectively), two were not indicated to have greying hair before age 40 (1016-1 and 1338-1), and one was too young for these to be reliable indicators of WS (1580-1, age 11). This last patient is very probably not affected with WS, but with a more severe progeria. Thus it seems that these criteria may be necessary, though not sufficient, to indicate the presence of mutations in the *WRN* gene. Hyaluronic acid levels were only tested in one patient; in the future we will request this test to complete the clinical file.

The presence of secondary signs varied, both among patients with mutations and those without. Incomplete data on earlier patients makes generalisation difficult, but the most frequent secondary symptom was irregular dentition (7/18), followed by diabetes or hyperglycemia (6/18) and flat feet (6/18), then hypogonadism, soft tissue calcification, or high voice (each 5/18). Diabetes is reported to be one of the more frequent findings in WS: four of the eleven patients with at least one mutation presented either Type II diabetes or glucose intolerance, five had normal glucose metabolism, and for two there was no information. None of the non-mutated patients was known to have diabetes, though two of five for whom we had information were glucose intolerant. The issue of irregular dentition merits further investigation, as minor irregularities are common in the general population and we did not assess the severity of the irregularity. Soft tissue calcification was the only recurring secondary symptom unique to mutation-bearing patients, with hearing loss and neuropathy also noted only in a patient with a mutation. Conversely, cardiomyopathy was found only in two patients without mutations and in none with mutations,

There is some indication that atypical progeria patients with dominant mutations in the *LMNA* gene tend to have a more severe course and associated cardiomyopathy than WS patients (Chen et al, 2003). This gene encodes the nuclear envelop proteins Lamin A and Lamin C, produced via alternative splicing. Dominant missense mutations of this gene are also responsible for other inherited syndromes of aging, notably the more severe Hutchinson-Guilford syndrome (HGS). Two of our patients are affected with cardiomyopathy, but no mutation in either *WRN* nor *LMNA* was found in either case. At this time, it is difficult to see how refinement of the clinical syndrome could better target the underlying gene, as patients positive for the cardinal symptoms of WS sometimes do not exhibit mutations in *WRN*, and the additional information given by secondary symptoms such as early atherosclerosis or osteoporosis is minimal.

Retrospectively, patient 1580-1 did not warrant analysis of *WRN*, and 1338-1 was analysed based on his alleged family history, not on the strength of his own symptoms. None of the other patients would have been excluded from analysis based on clinical signs. The weakest case was for 1019-1, though even if cardiomyopathy is eventually considered an excluding criterion, this patient exhibited all the cardinal signs except cataracts (for which we had no data).

The search for large rearrangements by quantitative PCR may reveal additional mutations in *WRN* both in our patients and in other populations. One such mutation, a deletion of 15 kb, is already listed in the University of Washington Werner Syndrome Database. A second strategy for finding the ‘missing’ *WRN* mutations is analysis of the mRNA, which could uncover deletions, duplications and insertions as well as defects in splicing due to mutations deep within the introns. A second blood sample should allow us to analyse *WRN* by RT-PCR as well as establish cell lines for functional and protein studies. Once this analysis is completed, we may have a more homogenous population in which to search for other genes involved in this rare and heterogeneous syndrome.

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