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***POLD1* Germline Mutations in Patients Initially Diagnosed with Werner Syndrome**

Davor Lessel^{1,*}, Fuki M. Hisama², Katalin Szakszon³, Bidisha Saha⁴, Alexander Barrios Sanjuanelo⁵, Bonnie A. Salbert⁶, Pamela D. Steele⁶, Jennifer Baldwin⁷, W. Ted Brown⁸, Charles Piussan^{9,†}, Henri Plauchu¹⁰, Judit Szilvássy¹¹, Edit Horkay¹², Josef Hoögel¹³, George M. Martin⁴, Alan J. Herr⁴, Junko Oshima⁴, and Christian Kubisch¹

¹Institute of Human Genetics, University Medical Center Hamburg-Eppendorf, Hamburg, Germany

²Division of Medical Genetics, Department of Medicine, University of Washington, Seattle, Washington

³Department of Pediatrics, University of Debrecen, Debrecen, Hungary

⁴Department of Pathology, University of Washington, Seattle, Washington

⁵Department of Medicine, Universidad Del Norte-CINPE Group, Barranquilla, Colombia

⁶Geisinger Medical Center, Danville, Pennsylvania

⁷Department of Dermatology, University of Minnesota, Minnesota

⁸New York State Institute for Basic Research in Developmental Disabilities, Staten Island, New York

⁹Pediatric Genetics, University of Amiens, Amiens, France

¹⁰Département de Génétique, Université Claude Bernard Lyon 1 et Hôpital Louis Pradel, Hospices Civils de Lyon F-69977, Bron CEDEX, France

¹¹Department of Oto-Laryngology and Head and Neck Surgery, University of Debrecen, Debrecen, Hungary

¹²Diagnoscan Hungary, Debrecen, Hungary

¹³Institute of Human Genetics, University of Ulm, Ulm, Germany

Abstract

Segmental progeroid syndromes are rare, heterogeneous disorders characterized by signs of premature aging affecting more than one tissue or organ. A prototypic example is the Werner syndrome (WS), caused by biallelic germline mutations in the Werner helicase gene (*WRN*). While heterozygous lamin A/C (*LMNA*) mutations are found in a few nonclassical cases of WS,

*Correspondence to: Davor Lessel, Institute of Human Genetics, University Medical Center Hamburg-Eppendorf, Martinistrasse 52, Hamburg 20246, Germany. d.lessel@uke.de.

†Deceased.

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another 10%–15% of patients initially diagnosed with WS do not have mutations in *WRN* or *LMNA*. Germline *POLD1* mutations were recently reported in five patients with another segmental progeroid disorder: mandibular hypoplasia, deafness, progeroid features syndrome. Here, we describe eight additional patients with heterozygous *POLD1* mutations, thereby substantially expanding the characterization of this new example of segmental progeroid disorders. First, we identified *POLD1* mutations in patients initially diagnosed with WS. Second, we describe *POLD1* mutation carriers without clinically relevant hearing impairment or mandibular underdevelopment, both previously thought to represent obligate diagnostic features. These patients also exhibit a lower incidence of metabolic abnormalities and joint contractures. Third, we document postnatal short stature and premature greying/loss of hair in *POLD1* mutation carriers. We conclude that *POLD1* germline mutations can result in a variably expressed and probably underdiagnosed segmental progeroid syndrome.

Keywords

mandibular hypoplasia; deafness; progeroid features (MDP) syndrome; *POLD1*; Werner syndrome

Introduction

The identification of mutations underlying monogenic segmental progeroid syndromes, which prematurely mimic physiological aging by simultaneously affecting multiple organs and tissues [Martin, 1978], has been instrumental in defining molecular pathways also involved in “physiological” human aging. A prototypic example is the Werner syndrome (WS), caused by biallelic loss-of-function mutations in the Werner helicase gene (*WRN*). WS is characterized by a variety of clinical signs of premature aging including skin atrophy, premature loss and graying of hair, bilateral cataracts, type 2 diabetes mellitus, atherosclerosis, osteoporosis, gonadal atrophy, and predisposition to various forms of cancer. We have previously shown that some patients initially diagnosed as having WS but without *WRN* mutations do have heterozygous mutations in *LMNA* [Chen et al., 2003; Hisama et al., 2011]. These patients—if compared with classical WS—display a somehow distinct phenotype, including an earlier age of onset and lack of the most penetrant feature of WS, bilateral ocular cataracts. These patients also differ from classical WS, in that they display somewhat more prominent lipodystrophic and cardiovascular abnormalities. Cancer is typically not described in these cases or in cases of Hutchinson-Gilford Progeria syndrome (HGPS), possibly because these latter patients typically die during the teenage years. Indeed, the literature makes reference to one in seven HGPS patients having died of a chondrosarcoma of the chest wall [Oshima et al., 1996] and another HGPS patient who developed an osteosarcoma [Shalev et al., 2007]. Further, we have shown that in about 10%–15% of patients initially diagnosed with WS, no causative mutations in either *WRN* or *LMNA* are found [Lessel et al., 2014; Oshima and Hisama, 2014]. These patients therefore provide an opportunity to identify new disease genes and molecular pathways involved in cellular and organismal human aging.

POLD1 (MIM #174761) belongs to a protein family of human DNA polymerases and possesses both polymerase and 3' to 5' exonuclease activities. It is responsible for DNA

synthesis of the lagging strand during DNA replication. *POLD1* functionally interacts with the Werner helicase during DNA replication and DNA repair [Kamath-Loeb et al., 2000; Szekely et al., 2000] and has already been implicated in human aging [Wang et al., 2012]. It was recently shown that heterozygous germline mutations in the exonuclease domain of *POLD1* predispose to colorectal [Palles et al., 2013] and endometrial [Church et al., 2013] cancers. Moreover, an in-frame single amino-acid deletion at the polymerase-active site of *POLD1* (c.1812_1814delCTC, p.Ser605del) was shown [Weedon et al., 2013] to cause mandibular hypoplasia, deafness, and progeroid features (MDP) syndrome [Shastri et al., 2010]. Additionally, a recent report identified a missense mutation (c.1519C>T, p.Arg507Cys) as a further cause of MDP [Pelosini et al., 2014]. These findings prompted us to specifically analyze the *POLD1* gene in patients referred to the International Registry of Werner Syndrome (<http://www.pathology.washington.edu/research/werner/registry/registry.html>) for whom we had previously excluded *WRN* and *LMNA* mutations as well as mutations in *BANF1* and *ZMPSTE24*, two genes that were reported to be also involved in segmental progeroid disorders [Agarwal et al., 2003; Puente et al., 2011].

Materials and Methods

Human Subjects

We evaluated 50 individuals who had been referred to the International Registry of Werner Syndrome with the putative diagnosis of WS, and in whom we had previously excluded *WRN*, *LMNA*, *BANF1*, and *ZMPSTE24* mutations. All biological samples and images were obtained following written informed consent from affected individuals or their legal representatives. The described clinical signs are based upon the clinical interpretations of referring physicians guided by published descriptions of the characteristic signs of segmental progeroid syndromes; however, due to the longtime of ascertainment, the contact to some of the patients or referring physicians has been lost so that we were not able in these cases to re-evaluate the clinical findings after the identification of the causative mutation. The study has ongoing approval from the University of Washington Institutional Review Board. The study was performed in accordance with the Declaration of Helsinki protocols.

Sanger Sequencing

DNA samples from whole blood were isolated by standard procedures. For gene analysis, we designed intronic primers to PCR amplify all coding exons and the exon-intron boundaries of *POLD1* by using genomic DNA of affected individuals. Primer pairs for the amplification of coding exons, and the approximately 50 bp of flanking UTR or intronic sequences, are available upon request. GenBank reference sequence, NM 001256849.1 (*POLD1*), was used for comparisons in all analyses. PCR products were directly sequenced on an ABI 3730 DNA Analyzer with BigDye chemistry v3.1 (Applied Biosystems, Foster City, California, USA). Sequence traces were assembled, aligned, and analyzed with the Seqman software (DNASTAR Lasergene, Madison, Wisconsin, USA). DNA mutation numbering system used is based on cDNA sequence with a “c.” symbol before the number.

Cell Lines

Epstein–Barr virus-transformed lymphoblastoid cell lines (LCLs) originating from *POLD1*-mutated patients (COLOM1010, JAE91301, and MINN1010), a WS patient (MY1010), and a healthy donor (AG1010) were established as previously described [Huang et al., 2006].

Chromosome Analysis

Exponentially growing LCLs were reseeded into culture flasks at a density of 2×10^5 /ml, left untreated for an additional 24 hr or incubated with either 40 ng/ml mitomycin C (MMC) for 48 hr or 0.3 μ M aphidicolin (APH) for 24 hr. Cells were exposed to 100 ng/ml colcemid for 2 hr, treated with hypotonic solution (NaCl/sodiumcitrate, 0.4%) for 15 min and fixed with 3:1 methanol–acetic acid. Slides were stained with Giemsa and 100 metaphase spreads were scored for chromosomal aberrations in two independent experiments. Metaphase spreads were observed using a Laborlux S microscope (Leitz, Wetzlar, Germany) and captured using CytoVision® (Applied Imaging, Grand Rapids, Michigan, USA).

Statistical Analysis

Data for DNA breaks in LCLs were compared (untreated, MMC, and APH) using Poisson regressions with log link functions with the number of observed metaphases as an offset variable. LCLs of AG1010 served as the reference. *P* values were not adjusted for multiple testing. *P* values, expressed as **P* < 0.05, ***P* < 0.01, and ****P* < 0.001, were considered to be significant.

Structural Modeling

The effect of mutations on POLD1 was visualized using the crystal structure of Pol δ from yeast [Swan et al., 2009a] (Protein Data Bank accession code 3IAY) in PyMol (The PyMOL Molecular Graphics System, Schrödinger, LLC.). POLD1 and the RB69 Pol editing complex [Shamoo and Steitz, 1999] (Protein Data Bank accession code 1CLQ) were aligned using the conserved DIE motif found in both exonuclease domains to position the 3' end of the primer strand from RB69 Pol in the exonuclease domain of POLD1. RB69 Pol and the other domains of POLD1 were removed for clarity and residues of interest were highlighted by representing them as spheres.

Results

Identification of *POLD1* Mutations in Cases Initially Diagnosed with WS

As mutations in *POLD1* were shown to result in a segmental progeroid syndrome [Weedon et al., 2013; Pelosini et al., 2014], and as POLD1 physically and functionally interacts with the Werner helicase, we screened the entire *POLD1* coding sequence in 50 *WRN* mutation-negative patients referred to the International Registry of Werner Syndrome with the putative diagnosis of WS. Direct Sanger sequencing of all coding exons and adjacent splice sites identified a recurrent heterozygous single amino acid in-frame deletion p.Ser605del [Weedon et al., 2013] in seven affected individuals, as well as the previously reported heterozygous missense mutation p.Arg507Cys [Pelosini et al., 2014] in one patient (Fig. 1). Thus, the molecular analysis allowed the diagnosis of MDP syndrome in these individuals.

No additional mutations were identified in the coding regions of the remaining 42 patients. Detailed clinical features of the eight *POLD1*-mutated patients as indicated by the referring physicians at the time of inclusion in the International registry of Werner syndrome are summarized below and in Table 1. In two patients, HD1010 and CP17900, we could confirm the in-frame deletion to be de novo, as it was not identified in the parents of both patients. Parental DNA or DNA samples from additional family members of the other patients were not available for genetic testing. Of interest, we were able to identify the first vertical transmission of a *POLD1* germline mutation in a segmental form of progeria (from mother [BRA90250] to her son [BRA90251]).

Werner Syndrome Registry HD1010

This Hungarian girl (Fig. 2) was referred to the Registry at the age of 10.5 years. She was born at 37 weeks of gestation with a birth weight of 3.3 kg. Her early developmental milestones and development were normal until the age of 4 years, when thin limbs and a wide trunk were observed. By the age of 6 years, mandibular underdevelopment was evident. At the age of 9 years, a round face, proptosis, a pinched nose, hypoplastic alae nasi, and a tented upper lip were noted. Further, a broad and sagittal long thorax, thin limbs and skin, and hypertrichosis of the calves were observed. Due to joint contractures, the patient was unable to make a fist or completely close her mouth. At the age of 10.5 years, thin skin and limbs, loss of facial subcutaneous fat, dental crowding with irregular teeth, joint contractures, mandibular underdevelopment, muscle wasting, and a high-pitched voice were observed. An audiologic examination revealed mild hearing loss of around 15 dB on the left ear and (still) normal hearing on her right ear. Diabetes, telangiectasia, osteopenia, or hypogonadism were not observed. She had high triglyceride values and C-peptide values, whereas levels of glucose, insulin, AST, ALT, and total bilirubin were all in the normal range (Table 1). No internal organ anomalies were observed by ultrasound. Her parents are healthy and the *POLD1* mutation was shown to have occurred de novo.

Werner Syndrome Registry COLOM1010

The clinical characteristics of this 17-year-old Colombian girl as well as photographs of this patient have been published in detail before [Barrios Sanjuanelo and Munoz Otero, 2010]. Briefly, she presented with short stature, truncal obesity, limb lipodystrophy, irregular dental development, thin hair, osteopenia, sclerodermalike skin changes, and mandibular underdevelopment. It is reported that her already deceased mother (whose DNA was not available for testing) had similar features, including lipodystrophy, sclerodermalike skin changes, and progeroid facial appearance characterized by mandibular underdevelopment, pinched nose, and tight facial skin with loss of subcutaneous fat. She died of ovarian cancer at the age of 34 years. Further clinical ascertainment revealed that this individual developed hearing impairment at the age of 11 years. Other family members have not been reported to have similar features.

Werner Syndrome Registry CP17900

This French woman was referred to the Registry at the age of 28 years. She presented with hearing impairment at the age of 12 years, followed by short stature, thin limbs, loss of

facial subcutaneous fat with mandibular underdevelopment, scleroderma-like skin changes, muscle wasting, and osteopenia. DNA analysis of the healthy parents revealed the deletion to be “de novo.”

Werner Syndrome Registry JAE91301

This woman of European ancestry was referred to the Registry at the age of 30 years. She was born at 37 weeks gestation with a birth weight of 2.41 kg. Her early developmental milestones and development were normal until the age of 8 years, when a telangiectatic rash developed on her hips, and spread to other parts of her skin. A skin biopsy revealed findings consistent with telangiectasia macularis eruptiva perstans. At that time, a widening of the trunk with loss of subcutaneous fat in the limbs was noted. Her menarche was at age 11, with normal pubertal development. She was diagnosed with hypothyroidism at age 14. At age 17, her skin was dry, with generalized poikiloderma, along with diffuse telangiectasias of the lower trunk and legs and callosities over the bony prominences of both feet. At age 14, she developed high nonfasting cholesterol and triglyceride values. Glucose, AST, GGT, total bilirubin, HDL, and LDL were all in the normal range. At age 15, she further developed a high random insulin level and a high C-peptide; hemoglobin A1C was normal. ANA autoantibodies were positive in a nucleolar pattern (Table 1). Abdominal ultrasound at age 17 did not show hepatomegaly. CT scans of the abdomen and pelvis performed at age 29 for abdominal pain showed fatty infiltration of the liver. Interestingly, though no audiometric examination was performed, the referring physician observed no hearing deficit. In addition, premature hair loss was observed. She had a 9-year-old son reported to have a large abdomen and thin arms and legs, and a 2-year-old presumably healthy daughter. She had no siblings. Her father's height was 155.5 cm; he had diabetes. Her mother's height was 171 cm; she was reported to have a Raynaud's phenomenon. The maternal grandmother died at age 42 of “gynaecologic” cancer. DNA samples and clinical information on further family members were not available.

Werner Syndrome Registry BRA90250 and BRA90251

This French woman was referred to the Registry at the age of 38 years; she presented with hearing loss, dental crowding, muscle wasting, scleroderma-like skin changes, loss of subcutaneous fat in the limbs, progeroid facial features characterized by mandibular underdevelopment, pinched nose, tight facial skin with loss of subcutaneous fat and premature hair greying. Her height was 148 cm. Her son, WS Registry BRA90251, was born with a birth weight of 2560 g. By the age of 10, he developed thin limbs with loss of subcutaneous fat, and skin atrophy. Both mother and son developed hearing impairments by the ages of about 14 years. This is to our knowledge the first case of a proven vertical transmission of this inframale amino-acid deletion. DNA samples and clinical information on further family members were not available.

WS Registry LONDO6010

This Swedish man was referred to the Registry at the age of 18 years. He presented with muscle wasting, thin arms and legs, and progeroid facial features characterized by mandibular underdevelopment, pinched nose, and tight facial skin with loss of subcutaneous fat. Based upon the clinical interpretations of the referring physician, no hearing deficit was

reported. Since the referring physician died 10 years ago, we were not able to recontact the family. DNA samples and clinical information on further family members were thus not available.

Werner Syndrome Registry MINN1010

This man of European ancestry was referred to the Registry at the age of 62 years after being seen for preoperative evaluation for toe amputation because of osteomyelitis. The patient had premature onset of hair graying when he was 20 years old. His medical history included: sensorineural hearing loss, eosinophilic fasciitis, episodes of cellulitis, and osteomyelitis of both legs, resulting in toe amputations, adrenal adenoma, a calcified left thigh nodule, depression, and anxiety. There was no history of cataracts. In his 40s, he was diagnosed with diabetes mellitus, and was treated for hypertension. He had a myocardial infarction at the age of 62 years. A cardiac catheterization revealed 70%–90% occlusion of his coronary arteries. An echocardiogram was notable for regional wall motion abnormalities, left ventricular hypertrophy, borderline low-ejection fraction, and mitral valve insufficiency. Coronary artery bypass surgery was scheduled, but his peripheral veins were too calcified; the surgery was therefore aborted. On physical examination at the age of 62 years, he was described as a short, slender man with truncal obesity and thin limbs whose height was 157 cm (<3rd centile), and whose weight was 66 kg (50–75th centile). He had progeroid facial features characterized by pinched nose, tight facial skin with loss of subcutaneous fat and gray hair. His voice was high-pitched and hoarse. His skin was tight, with hyperkeratosis of the soles and elbows, and blanching telangiectasias on the lower trunk. He had flat feet with chronic, bilateral ulcerations, and prior toe amputations. Interestingly, and in comparison to the other patients presented here, this person displayed no signs of mandibular underdevelopment. He was born to nonconsanguineous parents. His father died of coronary artery disease at 42 years, but his mother was alive and well at the age of 89 years. He had one adult healthy child and several grandchildren as well as three living brothers and three sisters with reported vascular disease and diabetes. One sister died at 61 years of age. DNA samples and clinical information on further family members were not available.

Structural Implications of POLD1 Mutations

The p.Ser605del mutant Pol δ , which lacks a highly conserved residue in the polymerase-active site (Fig. 1A), can bind to DNA but is not able to catalyze DNA polymerization [Weedon et al., 2013]. This contrasts with Pol δ variants encoded by cancer predisposing germline POLD1 alleles [Palles et al., 2013], which affect the exonuclease domain of POLD1 and increase base-substitution error rates. It was therefore postulated that germline mutations affecting the polymerase-active site increase replication stress, resulting in progeroid disorders, whereas mutations affecting the exonuclease domain increase mutation rates that lead to highly penetrant cancer susceptibility. The present study, our findings in Werner Registry patient MINN1010, and one previous report [Pelosini et al., 2014] might suggest that this hypothesis is an oversimplification. We therefore analyzed and compared the relative localizations of “progeroid” and “malignant” types of substitutions in the POLD1 protein structure by inferring their amino acid positions from the crystal structure of Pol δ from the yeast *Saccharomyces cerevisiae* [Swan et al., 2009a] (Fig. 3A). In the exonuclease domain, the binding pocket for the terminal nucleotide of the primer strand is

created by Pro327 (along with Phe326 and Phe401) on the left, Iso494 on the right, and below, by catalytic metal ions, coordinated by fully conserved carboxylate residues (Fig. 3B). The penultimate deoxynucleotide is held in place through stacking interactions with Phe401 and hydrogen bonding of the phosphate with the amino group of Leu474, which resides at one end of a short α -helix (α G). These interactions are altered by cancer-associated *POLD1* exonuclease substitutions. For example, the p.Pro327Leu substitution likely inserts an aliphatic chain into the binding pocket of the terminal nucleotide. p.Leu474Pro and p.Ser478Asn change residues along the interface between α G and a much longer α -helix (α I). This interface not only positions the α -amino group of Leu474 to interact with primer backbone, but also supports one of the catalytic carboxylate residues (Asp520). In contrast to the cancer-related substitutions within the exonuclease domain, p.Arg507Cys changes a highly conserved surface-exposed residue, well away from the exonuclease-active site (Fig. 3B). A disordered loop (486–491) in the vicinity of Arg507 prevents us from fully appreciating the impact of p.Arg507Cys; however, the path of the homologous loop from RB69 Pol suggests that the *POLD1* loop may interact with the basic guanido group of Arg507, helping to create the inside surface of the exonuclease domain that guides the primer strand into the active site. While it is certainly possible that disruption of this interaction induces a mild mutator phenotype, the disease clinical features suggest that the critical defect of the polymerase lies elsewhere. Given the distance of p.Arg507Cys to the polymerase-active site, it seems rather unlikely to alter polymerase activity as has been described for p.Ser605del. On the other hand, p.Arg507Cys could conceivably induce replication stress (like proposed for p.Ser605del) by allowing the primer strand to slip out of the back of the exonuclease domain. Extensive slippage may then create a stalled polymerase complex that triggers replication stress pathways.

Chromosomal Stability

It has been proposed that the putative increase of stalled replication forks due to progeria-related *POLD1* mutations may also lead to double-stranded DNA breaks, chromosomal instability, and cell senescence, which is also found in WS. To analyze this in more detail, we performed chromosome analyses in LCLs of patients with *POLD1* mutations under basal and stressed conditions. Interestingly, metaphase spreads prepared from LCLs of *POLD1*-mutated patients (COLOM1010, JAE91301, and MINN1010), revealed no chromosomal instability as compared with a healthy donor (AG1010) and a WS patient (MY1010) (Fig. 4). Moreover, we observed no significant increase in chromosomal aberrations of *POLD1*-mutated LCLs even when cells were treated with two replication-related genotoxic agents MMC and APH (Fig. 4).

Discussion

An in-frame heterozygous deletion at the polymerase-active site in exon 15 of *POLD1*, c.1812 1814delCTC (p.Ser605del) has been shown to cause a defined segmental progeroid syndrome: MDP syndrome [Weedon et al., 2013]. In addition, a heterozygous missense mutation (p.Arg507Cys) was recently identified in another case of MDP [Pelosini et al., 2014], currently adding up to altogether five published patients with *POLD1*-related MDP syndrome. In this study, we report seven patients—referred to the International Registry of

Werner Syndrome for a molecular diagnosis of WS—in whom we identified the same in-frame amino-acid deletion, and therefore provide further evidence that this is a hotspot mutation. Moreover, we identified the previously once described missense mutation (p.Arg507Cys) in one further patient affected by a segmental progeroid syndrome, thereby independently confirming the pathogenicity of this variant and also suggesting that this might represent a hotspot mutation (<http://www.lovd.nl/POLD1>). Neither of the two mutations were found in publicly available databases such as dbSNP, the 1000 Genomes data, Exome Variant Server (<http://evs.gs.washington.edu/EVS/>), or ExAC Browser (<http://exac.broadinstitute.org/>), precluding that they represent ultra rare polymorphisms. Therefore, the molecular analysis finally established the diagnosis of MDP in these previously unclassified cases of segmental progeria.

The clinical characteristics of the affected individuals reported here in general correspond quite well to what has been described before in *POLD1* mutation carriers, as is evident from the detailed comparison of clinical features in WS, MDP, and segmental progeroid patients with *POLD1* mutations presented in Table 2. By more than doubling the number of patients with segmental progeroid disorders caused by *POLD1* mutations, however, we are able to report some noteworthy differences in clinical symptomatology and to develop better data on the relative frequencies of specific clinical features. As a note of caution, it however has to be stated that the description of phenotypes is in some cases solely based on the clinical description of the referring physician and was not re-evaluated after the identification of the causative mutation. Apart from lipodystrophy, which was also the most prominent finding in the previously reported patients, all patients reported here developed scleroderma-like skin changes, progeroid facial features, and hypogonadism in males. Most of the patients also developed muscle wasting and dental crowding. Interestingly, compared with the previous reports, only three of our patients developed type 2 diabetes, and only one developed joint contractures, a finding identified in all previously reported MDP patients. We also describe a 30-year-old woman without subjective signs of hearing impairment (JAE91301), previously considered to be an obligatory clinical feature of MDP. Although a formal hearing test has not been performed in that patient and one cannot exclude the development of a clinically relevant hearing impairment at some future date, this observation suggests that hearing impairment may not be an invariable concomitant or early feature of *POLD1*-mutated patients. The finding that three of our patients developed a “hair phenotype” (i.e., premature hair loss in JAE91301, premature hair graying in BRA90250 and MINN1010), characteristic features of WS, further emphasizes the clinical variability of this disorder. Last, but not the least, most of the segmental progeroid patients with *POLD1* mutations showed a height below the median value, as determined by the Z score. Short stature is also one of the key features of WS (as well as of many other segmental progeroid syndromes). It is a feature, however, that has not been explicitly described before in *POLD1*-mutated MDP patients. In addition to broadening and revising the clinical symptomatology, we here present the first case of a molecularly proven vertical transmission, that is, from mother (BRA90250) to son (BRA90251), of *POLD1*-related segmental progeroid syndrome. In addition, COLOM1010 most likely inherited the mutation from her affected mother and JAE91301 (female) and MINN1010 (male) have altogether three children. Thus, these findings provide strong evidence that *POLD1* mutations in segmental forms of progeria do not have obligatory

effects upon fertility in females and perhaps not even in males, although we were not able to analyze and prove paternity of MINN1010.

Cancer predisposition is a key feature in many segmental progeroid syndromes, including the WS. It still remains unclear, however, whether patients with *POLD1*-mutated segmental progeroid syndrome might also have a higher risk for cancer, as is well-established for individuals with *POLD1* exonuclease domain mutations [Church et al., 2013; Palles et al., 2013]. It was reported that mice bearing heterozygous alterations affecting the *POLD1* polymerase-active site do not have an increased cancer risk but do have accelerated tumorigenesis [Venkatesan et al., 2007]. Interestingly, individual MINN1010, despite bearing the missense mutation in the exonuclease domain, did not develop cancer until a relatively advanced age (i.e., 62 years). Cancer was also not observed in a report of a 48-year-old woman with a heterozygous mutation in the exonuclease domain [Pelosini et al., 2014]. These clinical observations, although still limited, correspond quite well with our structural molecular modeling data, which highlight the differences in localization and thus probable functionality of progeroid and cancer-associated *POLD1* exonuclease mutations. Further support for this formulation are the results of our chromosomal analyses, as we were able to show that *POLD1* progeria-related mutations do not result in enhanced chromosomal instability in LCLs under basal conditions or after treatment with replication-related genotoxic agents, thereby again differentiating WS (with *WRN* mutations) and other segmental progeroid syndromes (with *POLD1* or *LMNA* mutations). A caveat in the interpretation of these results, however, is the possibility that chromosomal instability may be occurring in a variety of other somatic cell types. Moreover, two of the family members of the patients presented here, the probably affected mother of COLOM1010 and the maternal grandmother of JAE91301, died of gynaecologic cancer. Due to the relatively high frequency of these types of cancer, these findings may just be coincidental but clearly need to be investigated in additional patients, especially given that DNA of both individuals were not available for testing. Thus, the hypothesis that exonuclease domain substitutions cause cancer, whereas active site mutations trigger replication stress with progeroid features, should be reconsidered. The p.Arg507Cys substitution is not the only exception. Recent analysis of a brain tumor from a patient with biallelic MMR deficiency revealed an early mutation encoding an p.Leu606Met in the polymerase-active site that dramatically increased the mutation rate [Shlien et al., 2015]. Distinguishing between the two nonmutually exclusive roles of *POLD1* mutations, namely, enhanced carcinogenesis and premature aging, will require further work and will be the focus of future studies.

Conclusion

The eight patients presented here provide further support for *POLD1* mutations as a genetic cause of segmental progeroid syndromes. In addition, we expand the clinical spectrum associated with *POLD1* germline mutations in MDP syndrome and suggest that the genetic analysis of *POLD1* should be extended to patients with unclassified segmental progeroid syndromes.

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Figure 1.

Identification of causative *POLD1* mutations. **A:** Protein structure of *POLD1*. The position of the mutations identified in progeroid patients are marked with vertical arrows and shown in black; cancer-associated mutations are shown in red. (NN terminus; C-C terminus). **B:** Sanger sequencing chromatograms of parts of *POLD1* exon 13 after PCR amplification of genomic DNA. Sequencing results for MINN1010 bearing a heterozygous c.1519C>T (p.R507C) and an individual homozygous for the reference sequence (WT/WT). The amino acid translation is shown in the three-letter code above the chromatograms. **C:** Sanger sequencing chromatograms of parts of *POLD1* exon 15 after PCR amplification of genomic DNA. Sequencing results for HD1010 bearing a heterozygous c.1812_1814delCTC (p.Ser605del) and an individual homozygous for the reference sequence (WT/WT). The amino acid translation is shown in the three letter code above the chromatograms. Nucleotide numbering uses +1 as the A of the ATG translation initiation codon in the reference sequence, with the initiation codon as codon 1.

Figure 2. Clinical characterization of a patient with POLD1-mutated segmental progeroid syndrome (HD1010). **A:** Facial images from the age of 5 months until present. Note that starting from 9 years of age, the mouth can only be closed with extreme muscle power. **B:** From left to right: broad and sagittally long thorax, thin extremities, hypertrichosis of the calves at the age of 9 years. Maximum fist and white discoloration of the skin over the joints when bending the fingers at the age of 9 years is shown in the upper middle image. Dental crowding (lower middle image) is also shown on the left lateral X-ray. X-ray shows the underdeveloped mandible.

Figure 3.

Locations of Pol δ amino acids implicated in segmental forms of progeria. **A:** The Pol δ structure from *S. cerevisiae* is rendered as a ribbon diagram [Swan et al., 2009b] (Protein Data Bank accession code 3IAY). Amino acid positions are numbered by the corresponding human position. Progeria-associated amino acids (R507 and S605) are shown as teal CPK spheres. Polymerase domains and other structural elements are color coded: amino domain, gray; exonuclease (exo), red; palm domain, purple; fingers domain, blue; thumb domain, green; primer template DNA, tan sticks; dNTP, orange CPK sticks; catalytic carboxylate residues, green CPK sticks; a disordered loop (486–491) in the exonuclease domain, orange dashed line. The palm and fingers domains together form the polymerase catalytic active site. **B:** Location of R507C in the Pol δ exonuclease domain compared with cancer-associated amino acid substitutions. Superimposed on the Pol δ Exo domain are the last three nucleotides of the primer strand (yellow CPK sticks) and coordinating metal ions (green spheres) from the editing structure of the highly related polymerase, RB69 Pol [Shamoo and Steitz, 1999] (Protein Data Bank accession code 1CLQ). Cancer-associated substitutions are gray CPK spheres. Amino acids that mediate binding of the primer strand or interact with R507 are red CPK spheres. αG and αI are α -helices discussed in the text.

Figure 4.

Chromosomal stability in LCLs of *POLD1*-mutated patients. Shows the mean number of aberrations per cell observed in 100 metaphases of an unaffected individual (AG1010), two individuals bearing a p.605Sdel mutation (COLOM1010 and JAE91301), an individual bearing a p.R507C mutation (MINN1010), and a Werner syndrome patient bearing a mutation in *WRN* gene (MY1010). LCLs were untreated or treated either with 40 ng/ml of MMC (mitomycin C) or 0.3 μ M of APH (aphidicolin). Bar graphs summarize two independent experiments. *P* values are relative to AG1010 for each treatment (n.s., not significant, ****P* < 0.001).

Table 1
Clinical and Biochemical Characteristics of *POLD1*-Mutated Patients Presented Here

	Patient 1 JAE91301	Patient 2 CPT17906	Patient 3 COLOM1010	Patient 4 LONDO6010	Patient 5 BRA90250	Patient 6 BRA90251	Patient 7 HD1010	Patient 8 MINNI010
Sex	F	F	F	M	F	M	F	M
Age (years)	30	28	12	18	39	10	10	62
Ethnicity	US, mixed European	French	Columbian	Swedish	French	French	Hungarian	US, mixed European
Birth weight (kg) (Z score)	2.4 (−1.9)	2.65 (−1.5)	?	?	?	2.56 (−1.6)	3.30 (−0.2)	?
Height (cm) (Z score)	157.5 (−0.9)	157 (−1.5)	130 (−3.7)	162 (−1.95)	148 (−2.4)	123 (−2.2)	145 (1.03)	157 (−2.8)
Weight (kg) (Z score)	54.4 (−0.43)	34 (−5.0)	31 (−2.3)	39.1 (−4.5)	37 (−3.9)	21 (−2.78)	31 (−0.31)	66 (−0.4)
BMI (kg/m ²)	21.9	13.8	18.3	14.9	16.9	13.9	14.7	26.8
Metabolic Profile								
Diabetes (age at diagnosis)	Y (28y)	N	N	N	N	N	N	Y (43y)
Fasting glucose (70–110 mg/dl)	84 (1 hr post-prandial)	76	93	?	94	84 (1 hr post-prandial)	76	93
HbA1C (3.4%–6.0%)	4.7	Normal glucose tolerance test	?	?	Abnormal glucose tolerance test	?	5.6	?
Hepatic steatosis	Y	?	?	?	?	?	N	?
Abnormal liver function tests	N	?	?	?	?	?	N	N
ALT (5–35 U/l) AST (3–30 U/l)	32.21	?	44.43	?	?	?	30.26	25.30
Bilirubin (0.2–1.0 mg/dl)	0.8	?	0.74	?	?	?	0.33	0.3
Total cholesterol (120–170 mg/dl)	175	171	227	?	226	?	116	174
HDL (35–70 mg/dl)	27	47	?	?	?	?	31	25
Abnormal triglyceride ever	Y	Y	?	?	Y	N	Y	Y
Triglycerides (35–120 mg/dl)	215	190	?	?	218	Normal	212	232
Hypothyroidism (age at diagnosis)	Y (14y)	N	N	N	N	N	N	N
Urinary hyaluronic acid (0–77 µg/g creatinine)	220	85	?	?	880	420	?	?
Antinuclear antibody	1:320	1:500	?	?	?	?	?	?
Clinical features								
Tight skin	Y	Y	Y	Y	Y	Y	Y	Y
Telangiectasia (age at diagnosis)	Y (8y)	Y	Y	N	Y	N	N	Y
Mandibular underdevelopment	Y	Y	Y	Y	Y	Y	Y	N
Premature greying or loss of hair	Y (loss)	N	N	N	Y (graying)	N	N	Y (graying)
Dental crowding/Irregular teeth	N	Y	Y	N	Y	Y	Y	N
High-pitched voice	N	N	Hoarse	N	Hoarse	Y	Y	Y
Hearing impairment (age at diagnosis)	N	Y (12y)	Y (11y)	N	Y (14y)	Y (14y)	N	Y?
Muscle wasting	Y	Y	N	Y	Y	N	Y	Y

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	Patient 1 JAE91301	Patient 2 CPI7900	Patient 3 COLOM1010	Patient 4 LONDO6010	Patient 5 BRA90250	Patient 6 BRA90251	Patient 7 HD1010	Patient 8 MINN1010
Thin arms and legs with wide trunk (age at diagnosis)	Y (12y)	Y	Y	Y	Y	Y	Y (4y)	Y
Osteopenia	N	Y	Y	N	N	N	N	?
Hypogonadism (age at menarche)	N (11y)	N (13y)	N (12y)	N	N (15y)	Cryptorchidism	N	N
Children	2	?	-	?	1	-	-	1
Cognitive function	Normal	Normal	Normal	?	Normal	Normal	Normal	Depression, anxiety
Family history	Son, age 9 by report	N	Mother, by report (deceased)	N	Son (patient 6)	Mother (patient 5)	N	N
<i>POLD1</i> mutation	c.1812_1814 delCTC p.S605del	c.1812_1814 delCTC p.S605del	c.1812_1814 delCTC p.S605del	c.1812_1814 delCTC p.S605del	c.1812_1814 delCTC p.S605del	c.1812_1814 delCTC p.S605del	c.1812_1814 delCTC p.S605del	c.1519C>T p.R507C

Table 2

Comparison of Clinical Findings in POLD1-Mutated Progeroid Patients Presented Here, POLD1-Mutated Patients from Previous Studies, and Werner Syndrome Patients

	Our study	Previous studies	Werner syndrome
Clinical features			
Facial dysmorphisms			
Graying or loss of hair	3/8	0/5	Y ^a
Tight skin around cheeks and small nasal bones	8/8	5/5	N
Dental crowding/irregular teeth	5/8	5/5	N
Mandibular underdevelopment	7/8	5/5	Y ^a
Other findings			
Bilateral cataracts	0/8	0/5	Y ^a
Hearing impairment	5/8	5/5	N
High-pitched voice	5/8	4/4	Y
Short stature	7/8	1/5	Y ^a
Teleangiectasia	5/8	4/5	N
Muscle wasting	6/8	5/5	Y
Joint contractures	1/8	4/5	N
Thin arms and legs with wide trunk	8/8	5/5	Y ^a
Osteopenia	2/7	4/5	Y
Diabetes	2/8	4/5	N
Hypogonadism	1/2 males	3/3 males	Y
Abnormal cognitive function	1/7	0/5	N
Neoplasms	0/8	0/5	Y

^aIndicates a cardinal clinical feature for diagnosis of Werner syndrome.