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PARP10 deficiency manifests by infantile neurodegeneration and DNA repair defect

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Abstract

DNA repair mechanisms such as Nucleotide Excision Repair (NER) and Translesion Synthesis (TLS) are dependent on proliferating cell nuclear antigen (PCNA), a DNA polymerase accessory protein. Recently, homozygosity for p.Ser228Ile mutation in the PCNA gene was reported in patients with neurodegeneration and impaired NER. Using exome sequencing we identified a homozygous deleterious mutation, c.648delAG, in the PARP10 gene, in a patient suffering from infantile neurodegeneration. In agreement, PARP10 protein was absent from the patient cells. We have previously shown that PARP10 is recruited by PCNA to DNA damage sites and is required for DNA damage resistance. The patient cells were significantly more sensitive to hydroxyurea and UV-induced DNA damage than control cells, resulting in increased apoptosis, indicating DNA repair impairment in the patient cells. PARP10 deficiency joins the long list of DNA repair defects associated with neurodegenerative disorders, including ataxia telangiectasia, xeroderma pigmentosum, Cockayne syndrome and the recently reported PCNA mutation.

Keywords

DNA repair; neurodegeneration

INTRODUCTION

Maintenance of genomic integrity is fundamentally important for normal cell division and basic cellular biological processes. Defects in the maintenance of genomic integrity are characterized by DNA damage sensitivity, manifesting clinically by growth deficiency, neurodegeneration, immune deficiency and photosensitivity as seen in ataxia telangiectasia,

xeroderma pigmentosum, and Cockayne syndrome [1–3]. Genome integrity is maintained by several mechanisms such as Nucleotide Excision Repair (NER) which involves the excision of a ~30 nucleotides of DNA and the synthesis of a replacing DNA stretch, or Translesion Synthesis (TLS), which ensures completion of DNA replication even when the template DNA is damaged [4, 5]. In NER, the synthesis of the replacing stretch is dependent on a DNA polymerase accessory protein, named proliferating cell nuclear antigen (PCNA) [6]. PCNA is a homotrimeric DNA ring encircling and freely sliding along the DNA helix. It provides processivity to DNA polymerases and acts as protein recruitment and docking platform for numerous repair and replication-associated factors [7]. Besides NER, PCNA is also essential for TLS; TLS requires the engagement of specialized, low-fidelity DNA polymerases that are able to replicate damaged DNA by inserting nucleotides across DNA lesions [8]. While the damage is not technically repaired, its bypass ensures that DNA replication is completed and thus promotes genome integrity. We and others have previously shown that the activity of the TLS polymerases on damaged DNA is regulated through post-translational modification of PCNA by ubiquitin. When the replication machinery encounters a DNA lesion, PCNA becomes mono-ubiquitinated which results in an exchange from the normal replicative polymerase to one of the TLS polymerases that are able to replicate through the damage [7, 9, 10]. Similar to NER, this process is essential for maintaining genomic stability during development.

Homozygosity for p.Ser228Ile mutation in the PCNA gene was previously reported in patients with neurodegeneration, growth retardation, deafness and photosensitivity accompanied by atrophy of the cerebellar hemispheres and vermis. In cells of these patients transcription-coupled NER was perturbed secondary to impaired interaction of PCNA with DNA metabolizing enzymes [11].

We now report a novel genetic disorder in a PCNA associated enzyme in a patient who suffered from severe neurodegeneration. The data underscore the importance of PCNA for normal neurological development in human.

PATIENT AND METHODS

Patient II-3 was the third child of consanguineous parents of Palestinian origin (figure 1A). He was born at term following an uneventful pregnancy; the birth weight was 2700 gram and the head circumference was 31.5 cm. Global developmental delay with failure to acquire developmental milestones was noted since early infancy; at three years of age he was unable to roll over or maintain sitting position and would not reach for objects. He recognized his parents, smiled socially and had stranger anxiety. There was no speech acquisition. On physical examination at three years the head circumference was markedly low (38.5 cm, Z score -5.6), as were the weight (8 Kg, Z score -6.2) and length (81 cm, Z score -4.1). The patient had facial dysmorphism including long philtrum, micrognathia and wide alveolar ridge (figure 1C-D). The neurological examination revealed axial and peripheral hypotonia, head lag, globally reduced muscle strength (3/5), and hyperactive deep tendon reflexes (+3). Hearing and ophthalmic examination were normal. Brain MRI performed at three years of age revealed cortical atrophy and delayed myelination with paucity of white matter with both supra- and subtentorial involvement, which included the cerebellar hemispheres, vermis

and pons (figure 2). Other systems were not involved as indicated by the normal abdominal ultrasound, echocardiogram, and the results of general blood hematology and biochemistry. Specifically, he had no unusual infections and no skin sensitivity.

The parents and the two sisters were healthy.

METHODS

Whole exome analysis

Exonic sequences were enriched in the DNA sample of the patient using SureSelect Human All Exon 50 Mb Kit (Agilent Technologies, Santa Clara, CA). Sequences were determined by HiSeq2500 (Illumina, San Diego, CA) as 100-bp paired-end runs. Data analysis including read alignment and variant calling was performed by DNAnexus software (Palo Alto, CA) using the default parameters with the human genome assembly hg19 (GRCh37) as reference. Parental consent was given for DNA studies. The study was performed with the approval of the ethical committees of Hadassah Medical Center and the Israeli Ministry of Health.

Cell lines and protein techniques

Patient lymphoblasts, as well as control LCL721 lymphoblast cell line (ECACC No. 09071301, obtained from Dr. Kristen Eckert, Penn State Hershey College of Medicine) were grown in RPMI media with 10% FBS and 2mM Glutamine. HEK293T cells (ATCC No. CRL-3216) were grown in DMEM media with 15% FBS and 2mM Glutamine. For PARP10 knockdown, the Stealth siRNA oligonucleotide (sequence: GCCTGGTGGAGATGGTGCTATTGAT) was purchased from Invitrogen. As control, Stealth RNAi Negative Control (Invitrogen) was employed. Lipofectamine RNAiMAX (Invitrogen) was used for siRNA transfection. Cell extracts were performed as previously described [13]. Antibodies used for Western blot are: PARP10 (Novus NB100-2157), PARP1 (Cell Signaling Technology 9542S), γ H2AX (Santa Cruz Biotechnology sc-101696), Phospho-Histone H3 Ser10 (Cell Signaling Technology 9701S), Actin (Genetex gt5512).

DNA damage assays

For DNA damage sensitivity, Patient and control LCL721 lymphoblasts were exposed to DNA damaging agents Hydroxyurea (HU; 5mM) and Mytomycin C (MMC; 0.1 μ g/ml) for 6 days. Cellular viability following trypan blue staining was measured using the EVE automated cell counter (NanoEnTek), according to the manufacturer's instructions. Alkaline comet assays were performed using the CometAssay Kit (Trevigen 4250) according to manufacturer's instructions. Apoptosis Annexin V measurements were performed using the FITC Annexin V kit (Biolegend 640906) according to manufacturer's instructions, using a BD FACSCanto 10 flow cytometer.

RESULTS

Exome analysis of the patient yielded 49.5 million mapped reads with a mean coverage of X72. Following alignment to the reference genome (Hg19) and variant calling, we

performed a series of filtering steps under the hypothesis of a recessively inherited, rare, causal allele. These included removing variants which were called less than X8, were off-target, heterozygous, synonymous, had MAF>1% at ExAC (Exome Aggregation Consortium, Cambridge, MA, (URL: <http://exac.broadinstitute.org>)) or MAF>4% at the Hadassah in-house database. 22 variants remained, but after careful consideration of pathogenicity prediction, evolutionary conservation and segregation in the family (Table S1), we focused on Chr8:g. 145059605delAG, NM_032789.3:c.648delAG, p.(Thr216fs) in the *PARP10* gene. We verified the finding by Sanger sequencing and noted that the parents carried the variant and the two healthy sisters were homozygous for the WT allele (figure 1A-B). The variant was not carried by any of the ~44,000 individuals whose exome analyses were deposited at ExAC (accessed July 2016) and covered this gene, nor was it present in our in-house database (~800 Moslem-Arab exome analyses).

PARP10 encodes poly (ADP-ribose) polymerase family, member 10 (PARP10, NP_116178.2), a 1025 amino acid protein which we previously found to be required for DNA damage resistance [13]. In order to determine the pathogenicity of the mutation, we generated EBV-transformed lymphoblast cell line from the patient. Western blot experiments using anti-PARP10 indicated the absence of PARP10 protein in the patient cells (figure 3A); PARP10 expression was clearly observed in the control LCL721 lymphoblast cells. Confirming the identity of the Western blot band as PARP10, a band migrating at the same molecular height in 293T cell extracts was efficiently eliminated following treatment with PARP10-targeting siRNA (figure 3A).

Using siRNA-mediated knockdown of PARP10 in 293T and HeLa cells, we previously demonstrated that PARP10 depletion results in sensitivity to DNA damage [13]. To investigate if the PARP10 c.648delAG mutation confers DNA damage sensitivity, we exposed the patient and control LCL721 lymphoblasts to DNA damaging agents Hydroxyurea (HU; 5mM) and Mytomycin C (MMC; 0.1µg/ml) for 6 days. We then monitored cellular viability following trypan blue staining, using an automated cell counter. These experiments revealed that the patient cells were significantly more sensitive to DNA damage than control cells (figure 3B). Notably, the HU sensitivity phenotype was particularly pronounced. This is in line with our previous findings that PARP10 is required for TLS, as HU causes replication stress which engages TLS, while MMC causes mostly interstrand crosslinks for which TLS is only a minor repair pathway.

To further confirm these observations, we exposed the patient and control LCL721 cells to HU (5mM) and MMC (0.1µg/ml) for 24h and measured apoptosis using two different readouts. We first performed Annexin V staining and measured Annexin V levels by flow cytometry. The patient lymphoblasts showed increased Annexin V staining, indicating increased apoptosis, compared to control cells, particularly in response to HU treatment (figure 3C). Next, we performed Western blots on extracts of non-treated and DNA damage treated patient and control cells, using a PARP1 antibody. Cleaved PARP1 is a protein marker of apoptosis. Consistent with the results of the Annexin V staining, cleaved PARP1 was significantly increased in the patient compared to control cells, and the difference was more pronounced for HU-treated cells (figure 3D).

To confirm that the increased apoptosis was indeed due to a DNA repair defect, we measured the levels of phosphorylated H2AX (γ H2AX), broadly employed as a readout of DNA damage. When compared to the control cells, the patient cells showed increased γ H2AX, indicating increased susceptibility to DNA damage in these cells (figure 3E). The increase in γ H2AX signal was also observed in non-damaged cells, indicating that the patient PARP10-mutant cells are also unable to efficiently repair endogenous DNA damage. Moreover, we observed a marked decrease in phospho-Histone H3 levels in DNA damage-treated patient cells (figure 3E). Phospho-Histone H3 is a mitotic marker and DNA damage initiates a signaling cascade which arrest cells in G2. Thus, DNA damage generally results in reduced Phospho-H3 levels. The observed reduction in Phospho-H3 levels in the patient cells indicates increased DNA damage, further confirming a DNA repair defect in the PARP10-mutant patient cells.

Finally, we directly measured DNA damage in the patient and control cells using the alkaline comet assay, which detects single and double strand DNA breaks. For this experiment, we exposed cells to UV radiation ($100\text{J}/\text{m}^2$ analyzed 4h after) since UV is more efficient at generating DNA breaks following replication fork stalling and collapse compared to HU and MMC; moreover, we have previously shown that PARP10 is required for resistance to UV treatment. In this experiment patient cells showed a small but significant increase in comet tail length compared to control cells (figure 3F), indicating an accumulation of DNA breaks in these cells and further demonstrating the DNA repair defect conferred by PARP10 mutation.

DISCUSSION

Unrepaired DNA lesions can block polymerase progression, threatening the stability of replication forks and leading to DNA breaks [12]. PCNA is instrumental in a large number of diverse genomic stability mechanisms, most notably in efficient DNA repair; several DNA repair pathways including TLS, NER, mismatch repair and base excision repair employ PCNA to help DNA scanning, lesion recognition, and DNA repair synthesis [7]. The repertoire of PCNA functions is further expanded by PCNA post-translational modifications by ubiquitin and SUMO.

Recently, we identified the ADP-ribosyltransferase PARP10 as a novel component of the TLS machinery. PARP10 is a newly identified, and still mysterious, member of the poly-ADP-ribose polymerase (PARP) family [13]. Unlike its well-described cousin PARP1, PARP10 lacks ADP-ribose polymerase activity and instead can only transfer a single ADP-ribose molecule to substrates (a process termed mono-ADP-ribosylation or MARYlation) [14, 15]. We found that PARP10 is recruited by ubiquitinated PCNA to DNA damage sites, where its ADP-ribosyltransferase activity is essential to promote TLS polymerase engagement and DNA damage bypass [13]. Consequently, PARP10 knockdown results in cellular hypersensitivity to DNA damage, and DNA replication defects.

Here we report a novel genetic defect, PARP10 deficiency, in a patient with severe neurodegeneration. Using exome analysis we identified homozygosity for the c.648delAG mutation in the PARP10 gene which resulted in lack of PARP10 protein in patient

lymphoblasts. This was associated with sensitivity to replication stress which was induced by HU and therefore engages TLS, accumulation of DNA breaks following UV radiation and enhanced DNA damage-induced apoptosis.

While our previous work identified the catalytic activity of PARP10 as essential for PARP10 activity in DNA repair, the relevant substrate(s) of PARP10 for DNA repair remain mysterious. Possible substrates include PCNA itself, components of the PCNA ubiquitination reaction (such as the E3 ubiquitin ligase Rad18), TLS polymerases, or some as yet unidentified component of the TLS machinery. We speculate that the DNA repair defect observed in the patient cells is caused by loss of ADP ribosylation of these substrates.

The clinical phenotype of our patient consists of severe neurodegeneration manifesting by lack of acquisition of developmental milestones and cerebral and cerebellar atrophy. The increased DNA damage sensitivity observed in our patient cells is similar to the DNA repair defect previously associated with the neurodevelopmental disorders ataxia telangiectasia, xeroderma pigmentosum, Cockayne syndrome and the PCNA mutation [1, 3, 16]. We therefore propose that the neurodegeneration of our patient is attributed to PARP10 deficiency.

Both PCNA related disorders, the previously reported Ser228Ile mutated PCNA and the present PARP10 deficiency, are rather similar at the cellular level: While PCNA is essential for both DNA replication and DNA repair, the Ser228Ile mutation seems to specifically affect the DNA repair capacity of PCNA, and not its proliferation roles [17]. Similarly, the PARP10 deficient lymphoblasts grow normally and have normal DNA synthesis rates and doubling time (not shown) but their DNA repair capacity is markedly impaired. While we did observe that even under normal growth conditions, patient cells show increased apoptosis and reduced phospho-H3 levels indicating mitotic delay, these phenotypes are likely caused by inability to deal with spontaneous DNA damage.

The present report thus underscores the importance of PCNA for DNA repair and for central nervous system development.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Family pedigree, PARP10 genotype and patient photos. A-Family pedigree with genotyping of the c.648delAG mutation in the PARP10 gene. B-the chromatogram of the mutation, of a patient (upper panel), a carrier (middle panel) and a healthy control (lower panel) (deleted nucleotides are marked by asterisk in the lower panel). C, D – patient photos at 3 years of age. Note microcephaly, long philtrum, micrognathia and wide alveolar ridge.

Figure 2. Brain MRI at 3 years of age. T2-weighted axial (A), midsagittal (D) and T1-weighted (B, C) axial images. Note cortical atrophy (arrows) and delayed myelination (four-pointed stars) with paucity of white matter with both cerebral and cerebellar involvement (chevrons).

Figure 3.

Patient-derived PARP10 mutant lymphoblast cells show sensitivity to DNA damage. A. Western blot showing that PARP10 protein is absent in Patient-derived lymphoblasts. As controls, PARP10 is detectable in control LCL721 cells. PARP10 was also detected in 293T cells, but not after transfecting these cells with PARP10-targeting siRNA. B. Patient-derived PARP10 mutant lymphoblasts are sensitive to DNA damage compared to control LCL721 cells. Patient and control LCL721 lymphoblasts were treated with Hydroxyurea (HU; 5mM) and Mytomycin C (MMC; 0.1μg/ml) for 6 days. Cellular viability was assayed using trypan blue staining and viable cells were counted using an automated cell counter. The average of

4 independent experiments, with standard errors, is shown. Statistical significance was calculated using the TTEST (two-tailed, equal variance). C. Increased apoptosis in DNA damage-treated patient cells. Cells were exposed to HU (5mM) and MMC (0.1µg/ml) for 24h, and Annexin V staining was performed. Flow cytometry was used to quantify the mean Annexin V staining. The average of 3 independent experiments, with standard deviations, is shown. Statistical significance was calculated using the TTEST (two-tailed, equal variance). D. Increased PARP1 cleavage, indicating increased apoptosis, in DNA damage-treated patient cells. Patient and LCL cells were treated with HU (2mM) and MMC (0.1µg/ml) for 24h. Cells were lysed and extracts were analyzed by western blot with the indicated antibodies. E. Increased DNA damage and reduced mitotic progression in PARP10 mutant cells exposed to HU and MMC. Patient and LCL cells were treated with HU (2mM) and MMC (0.1µg/ml) for 24h. Cells were lysed and extracts were analyzed by western blot with antibodies against γH2AX (DNA damage marker) and phospho-H3 (mitotic marker). F. Alkaline comet assay showing increased DNA damage in PARP10-mutant cells exposed to UV radiation. Cells were analyzed 4h after exposure to 15J/m². In total, 78 and respectively 79 comet tails were measured, pooled from two independent experiments. Error bars indicate standard errors. Statistical significance was calculated using the TTEST (two-tailed, equal variance).