Site-directed Mutagenesis (Y52E) of POLH Affects Its Ability to Bypass Ultraviolet-induced DNA Lesions in HaCaT Cells

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ABSTRACT

*DNA polymerase eta (Pol η) is one of several Y family translesion synthesis (TLS) polymerases in humans and plays an important role in maintaining genome stability after ultraviolet (UV) irradiation, as it carries out error-free TLS at sites of UV-induced lesions. We performed site-directed mutagenesis of human polymerase η gene (Y52E), confirmed by sequencing, then cloned wild-type mutant and POLH genes into the eukaryotic vector pEG FPN1. After transfecting wild-type and mutant plasmids into HaCaT keratinocytes, we tested for UV-induced cis-syn cyclobutane pyrimidine dimer (CPDs) DNA lesions, and analysed cellular viability by MTT cell proliferation assay. The results showed that CPD levels decreased with empty vector control (EVC), wild-type POLH and Y52E-POLH over 48 hours post-UV irradiation with 0.1 mW/cm2 UVB for 15 minutes (*p *= 0.025). The rate in CPD reduction of mutant POLH was less than in wild-type POLH. Cell viabilities of all three groups increased over 48 hours after UV irradiation, with the increased rate in the wild-type being higher than for mutant protein (*p *= 0.046). We conclude that Y52E POLH has reduced capacity to bypass UV-induced DNA lesions in HaCaT cells.*

Keywords: DNA lesion bypass, DNA polymerase η, HaCaT keratinocytes, mutation, ultraviolet

La Mutagénesis de Sitio Dirigido (Y52E) del POLH Afecta su Habilidad para Reparar por Bypass Replicativo las Lesiones del ADN Inducidas por la Luz Ultravioleta en Células HaCaT

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RESUMEN

*La ADN polimerasa eta (Pol η) es una de las varias polimerasas de la familia Y en los seres humanos, que hace posible la síntesis de translesión (TLS), y desempeña un papel en el mantenimiento de la estabilidad del genoma luego de una radiación UV, ya que lleva a cabo TLS libre de errores en los sitios de las lesiones inducidas por rayos UV. Realizamos mutagénesis de sitio dirigido, del gen η (Y52E) de la polimerasa humana, confirmada por secuenciación, y entonces clonamos genes POHL y genes mutantes de tipo salvaje, transformándolos en un vector eucariótico pEGFP-N1. Después de transfectar los plásmidos de tipo salvaje y mutantes en queratinocitos HaCaT, realizamos pruebas buscando las lesiones dímero de pirimidina cis-syn ciclobutano (CPD) del ADN, inducidas por UV, y analizamos la viabilidad celular mediante ensayo de proliferación de células MTT. Los resultados mostraron que los niveles de CPD disminuían con el control de vector vacío (EVC), el gen POHL de tipo salvaje, y el POHL Y52E, en 48 horas de post irradiación de UV con 0.1 mW/cm2 UVB durante 15 min (*p *= 0. 025). La tasa de reducción del CPD del mutante POHL fue menor que en el POHL de tipo salvaje. Las viabilidades celulares de los tres grupos aumentaron a las 48 horas después de la irradiación con UV, con un incremento de la tasa del tipo salvaje con respecto de la proteína mutante (*p *= 0.046). Concluimos que el POHL Y52E ha reducido la capacidad para reparar por bypass replicativo las lesiones del ADN inducidas por la luz ultravioleta en células HaCaT.*

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Palabras claves: Bypass de la lesión del ADN, ADN polimerasa η, queratinocitos HaCaT, mutación, ultravioleta

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INTRODUCTION

The World Health Organization (WHO) computational models predict that a 10% decrease in the stratospheric ozone would add 300 000 non-melanoma and 4500 melanoma cases annually, worldwide. Ultraviolet (UV) light is a high risk factor for human skin cancer because of its strong genotoxic effects on DNA (1). DNA damage induced by UV (UVA, UVB) and even high energy artificial irradiation (UVC) are related mainly to pyrimidine dimerization (2). The cis-syn cyclobutane pyrimidine dimers (CPDs) and pyrimidine(6–4) pyrimidone photoproducts (6-4PPs) are the primary DNA damage products (3–8).

In responding to DNA lesions, organisms develop protective mechanisms that bypass lesions through translesion DNA synthesis (TLS), nucleotide excision repair (NER), base excision repair (BER), mismatch repair (MMR), double strand break repair (DSBR) and apoptosis (9–10). Translesion synthesis is carried out by special TLS DNA polymerases in a process that bypasses DNA lesions by incorporation of a nucleotide opposite to the lesion site (11–12). DNA polymerase eta (Pol η), an important TLS polymerase, promotes highly error-free replication across CPDs, which is induced by UV irradiation (13–14).

DNA polymerase eta belongs to the Y-family of polymerases, which consists of polymerases Rev1, polymerase ι, κ and η, and can accurately and efficiently bypass CPDs induced by UV irradiation (15–17). DNA polymerase eta is encoded by POLH, and loss of it results in the disease xeroderma pigmentosum variant [XPV] (18–20). Like other Y-family polymerases, Pol η contains four domains: palm, finger, thumb and little finger (21). The Pol η N-terminus contains five conserved motifs, motifs I–V, and nine highly conserved acidic residues (22, 23). Substitutions for Try-52, a highly conserved amino acid within the human DNA polymerase η finger domain, causes a reduction in UV-induced lesions in yeast (24). Therefore, we performed site-directed mutagenesis and replaced Try-52 with glutamic acid (Y52E), and measured HaCaT cell viability after UV irradiation.

Enzyme linked immunosorbent assay (ELISA) results indicated that Y52E-POLH decreased the rate of CPD relative to wild-type POLH and empty vector control (EVC) after irradiation with 0.1 mW/cm^2 for 15 minutes. MTT assay results exhibited increased cellular viability of all three groups over 48 hours of UV irradiation, with an increase in viability of the wild-type group relative to the mutant group. We conclude that the Y52E Pol η in HaCaT cells has reduced functionality and bypasses UV-induced DNA lesions.

MATERIALS AND METHODS

Reagents and materials

High Fidelity Platinum® Taq DNA Polymerase for polymerase chain reaction (PCR) was purchased from Life Technologies (Carlsbad, CA, USA) and nucleotides and restriction enzymes were obtained from New England Biolabs (Ipswich, MA, USA). Oligonucleotides were synthesized by the WM Keck oligonucleotide synthesis facility at Yale University (New Haven, CT, USA). RNA isolation and purification and Quanti Tect Reverse Transcription, QIA prep, Spin Miniprep, and QIAquick PCR purification kits were purchased from Qiagen (Gaithersburg, MD, USA). HaCaT keratinocytes were kindly supplied by Dr Jingwei Xie from Marshall University, and cultured in DMEM with 10% fetal calf serum, 1% penicillin and streptomycin at 37 °C in an incubator supplied with 5% CO_2. Vector pEGFP-N1 was kindly provided by Dr Shimin Chen (Yale University). FuGENE HD transfection reagent was purchased from Promega Corporation (Madison, WI, USA). OxiSelect UV-Induced DNA Damage ELISA Kit (CPD quantitation) was purchased from Cell Biolabs, Inc. (San Diego, CA, USA), and Vybrant® MTT Cell Proliferation Assay Kit was purchased from Life Technologies (Grand Island, NY, USA). Anti-DNA polymerase η and anti-beta actin antibodies were used for western blots (1/1000, Abcam, Cambridge, UK).

Site-directed mutagenesis and cloning into eukaryotic vector pEGFP-N1

Site-directed mutagenesis was used to generate Y52E using Platinum Taq DNA Polymerase and the following primers: 5′ CGGCATCATTGCCGTATCAGAGGAGGCTCGCGCGTT TGGCGTCA 3′ and 5′ TGACGCCAAACGCGCGAGCCT CCTCTGATACGGCAATGATGCCG 3′. The substitution mutation was confirmed by sequencing at the WM Keck oligonucleotide synthesis facility at Yale University, and BLAST used to compare DNA sequences (25). We selected pEGFP-N1 as the vector to transfect wild-type POLH and Y52E-POLH into HaCaT cells, cloning into Xhol and EcoRI sites. We then added restriction enzyme recognition sites (Xhol and EcoRI) to DNA amplification PCR primers: 5′ ccg CTCGAG ATGGCTACCGGCCAGGATC 3′ and 5′ ccg GAATTC TTATGAAGCCGAGAATTTCGTGGCACACAG GAACAGCATC 3′. Xhol- and EcoRI-digested pEGFP-N1 was ligated to Y52E-POLH and wild-type POLH using T4 DNA ligase at 16 °C overnight. Products were ligated into competent DH5α using QIA prep Spin Miniprep Kit (Qiagen). Transformants were confirmed as Y52E-POLH-pEGFP-N1 and POLH-pEGFP-N1 by single and double enzyme digestion.

Transfection and DNA polymerase η expression

Before transfection, we determined the optimal G418 concentration for selection of HaCaT transfected cells with pEGFP-N1 containing neomycin. G418 concentrations induced nontransfected HaCaT cell death of > 90% within five to seven days. Y52E-POLH-pEGFP-N1 and wild-type POLH-pEGFP-N1 were transfected into HaCaT cells using FuGENE HD transfection reagent. Transfection experiments were initiated when cells reached approximately 80% confluence, and incubated for 48 hours. Trypsinized adherent cells were cultured in selection medium, and replaced every two days for 11 days. Individual clones were transferred to 96-well plates, and cultures maintained in the selection medium. RNA expression was analysed by reverse transcription PCR (RT-PCR) using Quanti Tect Reverse Transcription kit (Qiagen), and PCR products observed with ImageJ software (National Institutes of Health, Bethesda, MD, USA) and quantified relative to β-actin using RT-PCR amplification primers: 5′CGCCGTTGTGCAGTATAAAA3′ and 5′AGC AGCAGATCTGGGCAC3′. DNA polymerase η was analysed by western blot using anti-DNA polymerase η body (1/1000, Abcam), and anti-beta actin antibody (1/1000, Abcam) as controls.

UV irradiation

Ultraviolet irradiation was applied by a TL20W/01 Philips UVB-Narrowband lamp and irradiance measured by a UV-radiometer (UV-VIS Radiometer RM-12, Opsytec Dr Grobel, Ettingen, Germany). Empty vector control-transfected HaCaT keratinocytes were exposed to UV at 0.1 mW/cm^2 and wild-type POLH and Y52E-POLH clones selected.

DNA lesion bypass assay

Ultraviolet-induced DNA damage ELISA kit (CPD Quantitation) is an enzyme immunoassay developed for rapid detection and quantitation of CPDs in DNA. DNA was extracted from HaCaT cells using DNeasy Blood and Tissue Kits (Qiagen) and denatured DNA diluted to 2 μg/ml in phosphate buffered saline (PBS). DNA samples (100 μl) were added to the wells of DNA high-binding plant buffer, and incubated overnight at 4 °C. DNA was washed twice with PBS and 150 μl of assay diluent added to each well and blocked for one hour. Assay diluent was added and anti-CPD antibody incubated for one hour. Samples were washed with 1X wash buffer; 150 μl of blocking reagent was added to each well, washed three times, followed by the addition of 100 μl secondary antibody-enzyme conjugate and incubated at room temperature for one hour. After adding 100 μl of substrate, the enzymatic reaction was terminated with 100 μl stop solution in each well and absorbance read at 450 nm. Cell viability was determined by Vybrant® MTT cell proliferation assays. First, a 12-mM MTT solution was prepared by adding 5 mg MTT to 1 mL of sterile PBS. Cell culture medium was then replaced with 100 μl fresh medium per well, followed by the addition of 10 μl 12 mM MTT stock solution per well. Samples were incubated at 37 °C for four hours, 50 μl dimethyl sulfoxide (DMSO) was added to each well with thorough mixing and incubation at 37 °C for 10 minutes and absor-bance read at 540 nm.

RESULTS

We used T7 sequencing primer and T7 reverse sequencing primer to confirm the POLH mutation at the 52nd amino acid position, derived from the TAC→GAG mutation (Y52E), and DNA and protein BLAST analysis to verify the T7 sequencing. Wild-type and Y52E-POLH were cloned in the eukaryotic vector pEGFP-N1 at Xhol and EcoRI sites (Fig. 1), and confirmed by single and double restriction endo-

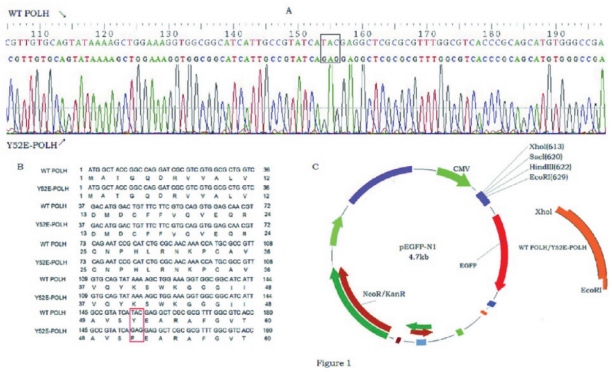

Fig. 1: Mutation and ligation confirmation. A: Comparison of wild-type and Y52E-POLH sequencing results show that they are the same except at site 154 and site 156. B: DNA and protein BLAST results of wild-type and Y52E-POLH showing substitution of tyrosine for glutamic acid at position 52. C: Restriction map of wild-type POLH1 and Y52E-POLH-pEGFP-N1 showing Xhol and EcoRI ligation sites.

nuclease digestion. We determined the minimum G418 concentration to use for transfections by titrating G418 from 0.2–0.8 mg/ml. HaCaT cells exhibited 0% viability by day nine at 0.6 mg/ml, with just a few cells viable at 0.5 mg/ml. Therefore, we chose 0.5 mg/ml as the selection concentration and cultured HaCaT cells in DMEM medium with 10% FBS and 1% penicillin and streptomycin. HaCaT cells were transfected in EVC, wild-type POLH-pEGFP-N1, and Y52E-POLH-pEGFP-N1, grown to approximately 80% confluence, and monitored by fluorescence microscopy (Fig. 2). Western blot analysis showed that HaCaT cells expressing wild-type and Y52E-POLH samples exhibited elevated expression relative to EVC transformants, consistent with the RT-PCR results ($p < 0.005$). We then examined HaCaT cell viability by MTT assay at different UV doses by controlling irradiation time (Fig. 3). The number of dead cells increased with increasing UV exposure, with Y52E-containing cells exhibiting the lowest viability (Fig. 3).

We tested CPD levels at different times after UV irradiation *via* ELISA and compared cell viabilities by MTT assay, measuring optical density at 540 nm. Cyclobutane pyrimidine dimer curves showed that EVC, wild-type POLH,

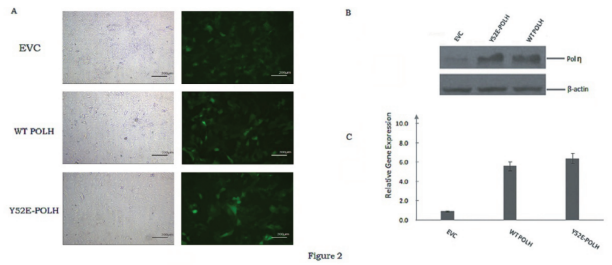

Fig. 2: Expression of empty vector control (EVC), Y52E-POLH, and wild-type POLH in transfected HaCaT cells. A: Microscopic (left, × 20, scale bar: 200 μm) and fluorescence images (right) of HaCaT cells transfected with EVC, wild-type POLH, and Y52E-POLH. B: Western blot analysis of DNA polymerase η expression. C: Relative RNA expression measured by reverse transcription polymerase chain reaction (RT-PCR): wild-type POLH and Y52E-POLH show elevated expression compared to EVC ($p < 0.001$).

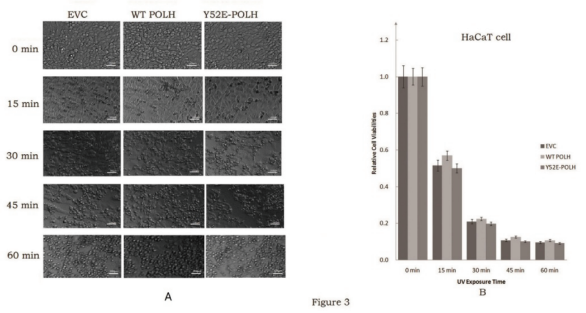

Fig. 3: Empty vector control (EVC), wild-type and Y52E-POLH transfected cells exposed to ultraviolet B (UVB) at 0.1 mW/cm^2 for different times (0, 15, 30, 45, and 60 minutes). A: Images of EVC, wild-type and Y52E-POLH transfected cells after UV irradiation (×20, scale bar: 200 μm). B: Relative cell viabilities analysed by MTT assay.

and Y52E-POLH groups decreased slowly over 48 hours, with CPD reduction of the mutant group slower than for the wild-type [$p = 0.025$] (Fig. 4A). Cell viabilities of all three groups increased over 48 hours after UV irradiation, with the wild-type group showing a more rapid increase than the mutant group [$p = 0.046$] (Fig. 4B).

DISCUSSION

We sought to determine whether the Y52E substitution in the DNA polymerase η gene interfered with Pol η-dependent TLS after UV irradiation. Translesion synthesis precedes thymine dimer formation by replicative DNA polymerase. Translesion synthesis polymerases lack proofreading activity and high processivity, but TLS polymerase processivity can be enhanced by proliferative cell nuclear antigen (PCNA), a trimeris complex encircling DNA, which prevents dissociation of DNA polymerases from DNA and provides a docking site for factors involved in DNA replication and repair (26, 27). Ultraviolet irradiation produces two predominant types of DNA lesions, CPDs and 6-4PPs. DNA polymerase eta

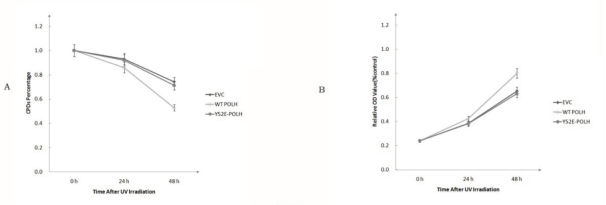

Fig. 4: A: Relative cyclobutane pyrimidine dimer (CPD) levels of empty vector control (EVC), wild type and Y52E-POLH transfected HaCaT cells after 0, 24, and 48 hours ultraviolet (UV) irradiation. B: Cell viability by MTT assay after 15 minutes with 0.1 mW/cm^2 UV irradiation. Y52E-POLH reduces CPD formation relative to wild-type POLH after irradiation with 0.1 mW/cm^2 UVB for 15 minutes ($p = 0.025$). Conversely, cell viability increased over 48 hours, with the rate of increase for wild-type POLH higher than for Y52E-POLH ($p = 0.046$). Data are mean ± SD of five experiments.

cannot bypass UV-induced PPs, which must be removed by NER proteins (28). Translesion synthesis induction of CPD by DNA polymerase η is a well-known process. Bypassing Pol η DNA lesions appears to be specifically adapted to synthesis of cis-syn CPDs (29). Tyr-52 is located in the "substrate lid" of the finger domain of Pol η, and the side chain of Try-52 faces into the active site cavity and may directly or indirectly affect interaction with incoming bases. Tyr-52 is a highly conserved amino acid in the Pol η finger domain, so Tyr-52 substitutions may affect human Pol η function. Biochemical analysis of active site mutations of human polymerase η shows that Q38, Y52 and R61 play key roles in determining polymerase fidelity (30), a key reason we chose Y52 as a substitution site. As a first step, we created the mutant Y52E-POLH, a single amino acid substitution in the active site, and cloned it into vector pEGFP-N1. We confirmed the Y52E mutation by sequencing pEGFP-N1 with T7 forward and T7 reverse primers, and confirmed orientation by restriction endonuclease digestion and green fluorescence protein expression *via* fluorescence microscopy.

Western blot and RT-PCR results indicate that wild-type POLH and Y53E-POLH exhibited strong expression. We then sought to determine UV-induced CPD production by MTT assay, and to estimate TLS by testing cell viability. Our results show that when UV irradiation increased, cell viabilities decreased, and that UV-induced DNA lesions are irreversible when UV dose increases beyond a certain threshold. Cyclobutane pyrimidine dimer levels were below wild-type POLH and EVC groups, and the rate of reduction of mutant POLH was slower than for wild-type and controls. Cyclobutane pyrimidine dimer levels increased immediately when DNA lesions were generated by UV irradiation, and decreased when DNA lesions were bypassed by TLS and other DNA repair mechanisms. Cyclobutane pyrimidine dimer levels decreased in EVC, wild-type POLH and Y52E-POL groups over 48 hours, with the rate of decreased CPD formation for the mutant group being slower than for the

other two groups. Conversely, cell viabilities of all the three groups increased over 48 hours, with the wild-type exhibiting greater viability than the mutant group.

The CPD levels and cell viability of these three groups suggest that the Y52E mutation impacts DNA polymerase η function *via* bypassing DNA lesions after UV irradiation. Further insights into the mechanism by which DNA polymerase η bypasses UV-induced DNA lesions may offer new therapeutic strategies for UV-induced skin cancer.

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