DNA Repair 11 (2012) 381–390

Contents lists available at SciVerse ScienceDirect

DNA Repair

journal homepage: www.elsevier.com/locate/dnarepair

The human gastric cancer-associated DNA polymerase β variant D160N is a mutator that induces cellular transformation

Katherine A. Donigan[a,b], Suzanne E. Hile[c], Kristin A. Eckert[c], Joann B. Sweasy[a,b,*]

[a] Department of Therapeutic Radiology, Yale School of Medicine, New Haven, CT 06520, United States
[b] Department of Genetics, Yale School of Medicine, New Haven, CT 06520, United States
[c] Department of Pathology, Gittlen Cancer Research Foundation, The Pennsylvania State University College of Medicine, Hershey, PA 17033, United States

ARTICLE INFO

Article history:
Received 5 October 2011
Received in revised form 4 January 2012
Accepted 5 January 2012
Available online 15 February 2012

Keywords:
Base excision repair
Cancer
Polymerase beta

ABSTRACT

Approximately 30% of human tumors sequenced to date harbor mutations in the *POLB* gene that are not present in matched normal tissue. Many mutations give rise to enzymes that contain non-synonymous single amino acid substitutions, several of which have been found to have aberrant activity or fidelity and transform cells when expressed. The DNA Polymerase β (Pol β) variant Asp160Asn (D160N) was first identified in a gastric tumor. Expression of D160N in cells induces cellular transformation as measured by hyperproliferation, focus formation, anchorage-independent growth and invasion. Here, we show that D160N is an active mutator polymerase that induces complex mutations. Our data support the interpretation that complex mutagenesis is the underlying mechanism of the observed cellular phenotypes, all of which are linked to tumorigenesis or tumor progression.

1. Introduction

Endogenous cellular DNA damage is estimated to occur upwards of 50,000 lesions per cell daily [1]. Left unrepaired, DNA lesions can give rise to genomic instability or mutagenesis. To guard against these consequences and maintain genomic integrity, cells have evolved various DNA repair mechanisms. The base excision repair (BER) pathway is responsible for resolving oxidative and alkylation damage, the predominant forms of endogenous DNA lesions [2]. BER is initiated by damage-specific glycosylases that recognize damaged bases, and the nature of the glycosylase determines which sub-pathway of BER the cell utilizes [3]. A monofunctional glycosylase will remove the damaged base, leaving an AP site that is then recognized by AP endonuclease (APE1). APE1 nicks the DNA backbone, leaving a 3′-OH and a 5′-deoxyribose phosphate (dRP) group. Pol β, having both polymerase and dRP lyase activity, binds this substrate, fills in the gap and removes the 5′-dRP group [4]. DNA ligase IIIα completes repair by sealing the nick [5]. Bifunctional glycosylases can remove the damaged base and nick the DNA backbone, resulting in a 3′-dRP group and a 5′-phosphate [6]. APE1 modifies the DNA by removing the 3′dRP group. BER again completes by Pol β filling in the gap and DNA ligase IIIα sealing the nick. The NEIL DNA glycosylases initiate an APE-independent BER subpathway, excising the damaged base and incising the DNA strand to leave 3′ and 5′-phosphate groups [7]. Polynucleotide kinase modifies this gap by removing the 3′-phosphate. Pol β binds this substrate and catalyzes incorporation of the correct dNTP opposite the templating base, followed by DNA ligase IIIα sealing the nick to complete repair. Regardless of the type of damage or BER subpathway initiated, Pol β is the main polymerase that is responsible for gap-filling DNA synthesis.

If any component of this multi-step BER process is not functioning properly in cells, DNA lesions or BER intermediates may persist. This can result in genomic instability or mutagenesis, hallmarks of tumorigenesis and tumor progression. First proposed in 1974, the mutator phenotype hypothesis indicates that the mutation rate observed in tumors is too high to be accounted for by the somatic mutation rate alone [8]. Defects in DNA replication or repair are thought to underlie the elevated mutation rate observed in human tumors [9]. Such defective pathways have indeed been identified in human tumors, including mismatch repair, methylation reversal repair and BER [10]. Tumor-specific Pol β variants have been identified in 30% of 189 tumors characterized to date [11]. Several non-synonymous amino acid substitution variants have functional

Abbreviations: Pol β, DNA polymerase β; BER, base excision repair; AP, apurinic/apyrimidinic; APE1, AP endonuclease 1; dRP, deoxyribose phosphate; NEIL, Nei-like; WT, wild-type; Tet, tetracycline; MEF, mouse embryonic fibroblast; Hyg, hygromycin; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; DTT, dithiothreitol; PBS, phosphate buffered saline; FPLC, fast performance liquid chromatography; dNTP, deoxynucleotide triphosphate; k_{obs}, observed rate constant; k_{ss}, steady-state constant; K_D, equilibrium dissociation constant; WCE, whole cell extract; UDG, uracil DNA glycosylase; CD, circular dichroism; XRCC1, X-ray repair cross complementing 1; FUdR, 5-fluoro-2′-deoxyuridine; Cm, chloramphenicol; HSV-*tk*, Herpes simplex virus-thymidine kinase.

* Corresponding author at: Hunter Radiation Therapy Center, P.O. Box 208040, New Haven, CT 06520-8040, United States. Tel.: +1 203 737 2626; fax: +1 203 785 6309.
E-mail address: joann.sweasy@yale.edu (J.B. Sweasy).

1568-7864/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
doi:10.1016/j.dnarep.2012.01.004

phenotypes that include polymerase deficiency, dRP lyase deficiency and subtle mutagenesis *in vivo* and *in vitro* [12–15]. Three of these variants, Glu295Lys, Ile260Met and Lys289Met, have also been shown to induce cellular transformation when expressed in cells [13,16]. These variants are found in the fingers sub-domain of Pol β, a region that contains residues that form the dNTP binding pocket and influence dNTP selectivity. The variant D160N was first identified in a small-scale screen that was focused upon sequencing *POLB* in 20 human gastric tumors [17]. Six gastric tumors were found to harbor a *POLB* mutation that would result in a unique non-synonymous single amino acid substitution. The previously described dRP-lyase domain variant Leu22Pro and fingers domain variant Glu295Lys were also identified in this screen. Residue D160 is found in the palm sub-domain, where the catalytic residues reside.

In this study, our goal was to determine if the gastric tumor-associated variant D160N exhibited altered biochemical properties and if cellular effects of D160N expression are linked to cancer etiology. In the work presented here, we provide evidence that D160N is an active polymerase that induces complex errors and that when expressed in cells, induces a variety of phenotypes related to cancer etiology. Taken together, our results are consistent with the interpretation that the D160N variant has a functional phenotype related to genomic instability and tumorigenesis.

2. Materials and methods

2.1. Plasmid DNA constructs and cloning

For protein purification, the wild-type (WT) Pol β cDNA was cloned into the pET28a vector containing an N-terminal 6X-histidine tag as previously described [18]. The D160N variant was generated using the QuickChange site-directed mutagenesis kit according to the manufacturer's instructions (Stratagene) with the following DNA primers:

5′-GAGGAGATGCTGCAAATGCAGAACATTGTTCTTAATGAAGTTA-3′ and 5′-TAACTTCATTAAGAACAATGTTCTGCATTTGCAGCATCTCCTC-3′ (Invitrogen). Introduction of the desired mutation was confirmed by direct sequencing at the Keck DNA Sequencing Facility at the Yale University School of Medicine. For retroviral infection into mouse cells, the WT Pol β cDNA with a C-terminal hemagglutinin (HA) tag was cloned into the pRVYtet retroviral vector as previously described [16]. The pRVYtet vector contains a selectable hygromycin resistance gene driven by an internal SV40 promoter and drives expression of Pol β proteins in a Tetracycline (Tet)-repressible manner from the tetO/CMV promoter. The pVSVG vector (Clontech) expresses an env glycoprotein that is used to generate retrovirus using the GP2-293 packaging cell line system (Clontech).

2.2. Cell lines and cell culture

C127 cells are non-transformed epithelial cells generated from a murine mammary carcinoma of an RIII mouse and purchased from ATCC [19]. C127 cells were grown at 37 °C in a humidified 5% CO_2 incubator and maintained in DME10 (Dulbecco Modified Eagle's Medium supplemented with 10% fetal bovine serum, 1% penicillin–streptomycin). The mouse embryonic fibroblast (MEF) cell line deficient in Pol β (88Tag Pol β−/−) was a gift from Leona Samson (Massachusetts Institute of Technology). Pol β-deficient MEFs were maintained in DME10 supplemented with 1% L-glutamine (Invitrogen) and grown at 37 °C in a humidified 5% CO_2 incubator. GP2-293 cells (Clontech) used for viral packaging were maintained in DME10 supplemented with 1% L-glutamine and 1 mM HEPES (Invitrogen) and grown at 37 °C in a humidified 5% CO_2 incubator.

2.3. Transfection, infection and expression analysis

To generate retrovirus containing the D160N or WT Pol β construct, the GP2-293 packaging cell line was co-transfected with the pRVYtet and pVSVG plasmids using calcium phosphate [16]. GP2-293 cells were incubated for 72 h before virus was harvested. C127 cells were infected with retrovirus supplemented with 4 μg/mL polybrene (American Bioanalytical) as described [16]. Stable clones were selected with 300 μg/mL Hygromycin (Hyg, Invitrogen) and 2.5 μg/mL Tet to suppress expression. Exogenous expression was evaluated by Western blot as described [16]. Briefly, clones were split and grown in the presence or absence of Tet until they were 80% confluent. Cell lysates were harvested by scraping with heated SDS loading buffer (50 mM Tris pH 6.8, 100 mM DTT, 2% SDS and 10% glycerol) and were boiled for 10 min and resolved on a 10% SDS-PAGE gel. Proteins were transferred to a nitrocellulose membrane using a semi-dry transfer apparatus and probed with monoclonal anti-Pol β antibody (Abcam 1831) at 1:250 dilution. Following extensive washing in 1× PBS supplemented with 0.1% Tween, the membrane was incubated with horseradish peroxidase-conjugated secondary antibody at a 1:1000 dilution. Bands were developed using ECL plus (Amersham) and exposed to film. Only clones exhibiting approximately equal levels of exogenous and endogenous Pol β expression were selected for subsequent assays. Bands were quantified using ImageQuant software.

2.4. Focus formation assay

To determine if expression of D160N results in a loss of contact inhibition, C127-D160N clones were passaged as previously described [16]. Briefly, cells were passaged every 3–4 days in DME10 supplemented with 300 μg/mL Hyg with or without Tet where appropriate. Every fourth passage, 1×10^4 cells were seeded into T25 flasks and cells were fed every 3–4 days with DME10 supplemented with 300 μg/mL Hyg, with or without Tet. After 25 days flasks were stained with Giemsa to visualize foci. Resulting foci were counted by microscopic examination. Flasks containing greater than 350 foci were deemed to have too many foci to be accurately counted. To determine if the presence of foci is related to a heritable change, D160N expression was extinguished at later passages by adding Tet back to the growth media and continued monitoring of foci as described.

2.5. Anchorage-independent growth assay

D160N clones were evaluated for the ability to grow in soft agar as described [16]. Approximately 1×10^5 late-passage cells grown under inducing or non-inducing conditions were mixed with DME10 supplemented with 0.3% Difco Noble agar and Tet if appropriate. The resulting mixture was poured onto 60 mm dishes containing DME10 and 0.6% Difco Noble agar. Cells were fed twice a week with 1 mL of DME10 supplemented with 0.3% Difco Noble agar with or without Tet where appropriate. After 5 weeks, colonies were visualized by light microscopy, with 10 fields from each of 5 plates per treatment scored for colonies. The number of colonies per field (±SE) reported is the average across all plates and $^*p < 0.01$ for the paired, two-tailed *t*-test.

2.6. Proliferation assay

To determine the effect of D160N or WT Pol β expression on cellular proliferation, late-passage cells were seeded at 2×10^4 cells per 60 mm dish with or without Tet as appropriate and allowed to attach overnight at 37 °C in a humidified 5% CO_2 incubator. A replicate of each plating condition was trypsinized and counted every 24 h for four days using an automated cell counter (Nexelcom).