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TITLE: Thymine DNA Glycosylase as a Novel Target for Lung Cancer

PRINCIPAL INVESTIGATOR: Alfonso Bellacosa, M.D., Ph.D.

CONTRACTING ORGANIZATION: Institute for Cancer Research
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Institute of Fox Chase Cancer Center 333 Cottman Avenue
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14. ABSTRACT

The purpose of this research is to develop novel therapeutics for lung cancer (LC) based on targeting the dual DNA repair and epigenetic factor Thymine DNA Glycosylase (TDG). In principle, inhibiting TDG may both disrupt DNA repair and the cancer epigenetic state of LC cells, thus achieving a therapeutic effect that enhances the actions of existing chemotherapies and is complementary to current epigenetic therapy agents.

In the current reporting period, we collected triplicate DNA and RNA samples from control and TDG knockdown A549 and H1299 lung cancer cells, in preparation for methylome and transcriptome studies. We also discovered that cells depleted of TDG have a phenotype of altered genomic stability involving mitotic function, which was completely unpredicted. Based on this discovery, in future experiments, we will evaluate the cytotoxicity of lung cancer cells treated with first-generation TDG inhibitors, as originally proposed, but in combination with anti-mitotic drugs.

15. SUBJECT TERMS
DNA methylation, DNA demethylation, DNA repair, epigenetic therapy, Thymine DNA Glycosylase, cancer cell killing, single-agent therapy, combination therapy, combination index

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Table of Contents

DOD Annual Report

Principal Investigators: Alfonso Bellacosa & Timothy Yen
Institution: The Research Institute of Fox Chase Cancer Center
Grant Number: W81XWH-17-1-0136

INTRODUCTION: The purpose of this research is to develop novel therapeutics for lung cancer (LC) based on targeting the dual DNA repair and epigenetic factor Thymine DNA Glycosylase (TDG). In principle, inhibiting TDG may both disrupt DNA repair and the cancer epigenetic state of LC cells, thus achieving a therapeutic effect that enhances the actions of existing chemotherapies and is complementary to current epigenetic therapy agents.

KEYWORDS: DNA methylation, DNA demethylation, DNA repair, epigenetic therapy, Thymine DNA Glycosylase, cancer cell killing, single-agent therapy, combination therapy, combination index

ACCOMPLISHMENTS:

What were the major goals of the project?

The major goals of this project are: (Specific Aim 1) to characterize the mechanisms by which TDG inhibition facilitates growth arrest, senescence and cell death; and (Specific Aim 2) to explore candidate TDG inhibitors as single agents and in combination with existing chemotherapies and epigenetic therapies.

What was accomplished under these goals?

We have previously shown that *TDG* knockdown in melanoma cell lines produces an anti-cancer effect characterized by reduced proliferation and decreased clonogenic capacity. We are happy to report that the melanoma studies are now published and this grant is acknowledged (Mancuso et al. *Oncogene* 38:3710-28, 2019). This effort was important because it generated new information that is currently being applied to characterize the mechanism by which TDG knockdown kills lung cancer cells (H1299 and A549) in vitro (see below).

As proposed in Aim 1, we used lentivirus to transduce cells with a shRNA that targeted TDG. Our previous progress report established knockdown by western blots, as well as loss of viability by clonogenic assay. In the current reporting period, we verified the clonogenic data by repeating the TDG knockdown experiments (data not shown). We then followed up on mechanistic studies to identify possible mechanisms of cell killing. One direction is to extract the DNA from control and TDG knockdown A549 and H1299 cells (in triplicates) and compare the methylome changes using methods described in our melanoma paper. In parallel, we extracted RNA so that we can perform RNAseq to compare the transcriptomes of the control and TDG knockdown cells. For both DNA and RNA extractions, we had to first confirm by western blots that the TDG was efficiently knockdown. We have just completed collection and extraction of the triplicate samples whose TDG levels were verified to be knocked down. These samples will be processed for methylome and transcriptome analysis.

Separately from the "omics" analysis, we followed up on a new observation that was made in TDG knockdown melanoma cells. We unexpectedly discovered that cells depleted of TDG lacked the presence of a set of proteins that specifically bind to centromeres on chromosomes. Western blots showed no changes in levels of expression before or after TDG knockdown. This demonstrates that the defect is due to recruitment of these proteins to the centromere. Given that loss of centromere function is established to cause cell death or senescence, two outcomes we have observed, we have focused our efforts to characterize this defect. CENP-B is a DNA sequence-specific binding protein that binds to a consensus element within the alpha-satellite DNA repeats that are exclusively found at centromeres of human chromosomes. Early studies showed that the 17bp binding site for CENP-B has CpG sites that are normally unmethylated and thus permissive for CENP-B binding. We are using a portion of the DNA that

we have extracted from control and TDG knockdown cells to analyze the methylation content of a representative alpha-satellite repeat. Based on preliminary analysis of methylation by bisulfite sequencing, we believe that when TDG is knockdown, the CpG sites become re-methylated, which has been shown to block binding by CENP-B. Indeed, we saw increased methylation in TDG knockdown samples, as predicted (**Fig. 1**). This increased methylation would render the otherwise functional centromere completely or partially inactive. Importantly, the methylation status at an adjacent *inactive* alpha satellite repeat was unchanged regardless of TDG knockdown (**Fig. 1**). This demonstrates that TDG is important for maintaining unmethylated CpG sites at active alpha satellites to allow CENP-B binding. Furthermore, the selectivity of TDG for certain CpG sites suggests it epigenetically marks active versus inactive alpha satellite repeats within the centromere. To maximize a potential effect, we knocked down TDG in combination with TET1, which is upstream in the demethylation pathway, in two lung cancer cell lines, A549 and H1299. Indeed, immunofluorescence staining comparing control and TET1-TDG knockdown cells show reduction/loss of CENP-B foci which reflects lack of CENP-B at centromeres (**Fig. 2**). The normalized foci count data were as follows: A549 control = 1.0, A549 TET1-TDG siRNA = 0.50; H1299 control = 1.0, H1299 TET1-TDG siRNA = 0.74.

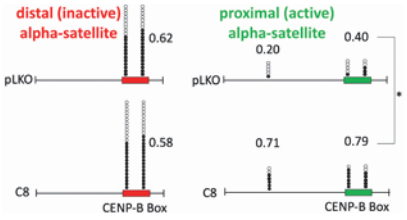

Figure 1. Methylation of distal and proximal centromeric CENP-B boxes in MEL501 cells infected with control lentiviral vector (pLKO) or TDG shRNA lenti (C8). Open/closed circles are unmethylated/methylated CpG sites. Fractions of methylated sites are indicated. * p=0.05

We are now systematically analyzing the methylation of CpG sites in the CENP-B consensus element to verify that the loss of staining correlates with changes (remethylation) of the DNA. The dependence of centromere integrity on TDG function in cancer cells was not anticipated, and as such was not included in our grant proposal and statement of work, but we believe disruption of centromere integrity is a major mechanism by which loss of TDG decreases cell viability, including viability of lung cancer cells, establishing relevance of these DOD-funded studies.

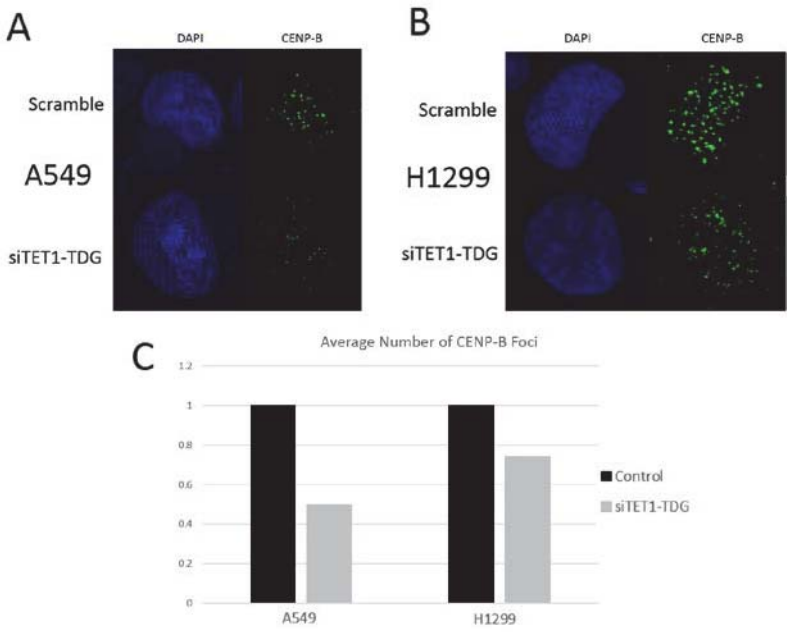

Figure 2. Indirect immunofluorescence staining of CENP-B in A549 (A) and H1299 (B) lung cancer cell lines, showing diminished staining pattern and decreased number of foci.

Because of the efforts directed towards characterizing the centromere defects associated with loss of TDG, we delayed the proposed studies testing clonogenic survival of cells treated with chemotherapeutic agents with and without TDG expression. The new discovery regarding CENP-B is directly relevant to the chemotherapeutic studies as it is important for mitosis. For this reason, we will change our drug treatment protocol: we plan to now test TDG knockdown in combination with sublethal doses of anti-mitotic drugs, such as paclitaxel. We will still include the first generation TDG inhibitors juglone in our combination studies, as originally proposed.

What opportunities for training and professional development has the project provided?

Dr. Rahul Prasad is a trained surgeon who decided to take a two-year break in his residency program and accept a postdoctoral position at Fox Chase Cancer Center, to become more proficient in cancer biology

and molecular genetics. This DOD award provided such a training opportunity, as Dr. Prasad was exposed to a variety of molecular biology techniques, including bisulfite sequencing for analysis of DNA methylation, and immunofluorescence, for analysis of centromeric proteins. Dr. Prasad's ultimate objective of professional development is to become an academic pediatrician with strong collaborations with translational basic science researchers; working on this project allowed Dr. Prasad to see first-hand how scientific collaborations between clinicians and basic scientists are established, and to build a network of colleagues interested in cancer research and cancer care that will be most beneficial to him in the future when he will transition to scientific independence.

How were the results disseminated to communities of interest?

Nothing to Report.

What do you plan to do during the next reporting period to accomplish the goals?

In the next reporting period, we plan to publish the results showing that TDG knockdown causes cell cycle arrest and senescence of LC cell lines. We also plan to assess the mechanisms by which TDG knockdown has such anti-cancer effects on LC cell lines, to complete Aim 1. Finally, for the final report, we plan to investigate the role of TDG inhibitors as single agents or in combination with agents used in the clinic against LC, to complete Aim 2.

IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

The dependence of centromere integrity on TDG function in lung cancer cells is an important DOD-funded discovery that expands epigenetic control beyond transcriptional regulation, and will clarify how epigenetic principles and factors used for transcriptional regulation by DNA methylation (writers/erasers/readers) are employed to maintain centromere stability and achieve chromosomal stability. In addition, this discovery is important to devise strategies for killing lung cancer cells with inhibitors of TDG.

What was the impact on other disciplines?

Nothing to Report.

What was the impact on technology transfer?

Nothing to Report.

What was the impact on society beyond science and technology?

Nothing to Report.

CHANGES/PROBLEMS:

Changes in approach and reasons for change

Nothing to Report.

Actual or anticipated problems or delays and actions or plans to resolve them

Nothing to Report.

Changes that had a significant impact on expenditures

Nothing to Report.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Nothing to Report.

Significant changes in use or care of human subjects

Nothing to Report.

Significant changes in use or care of vertebrate animals

Nothing to Report.

Significant changes in use of biohazards and/or select agents

Nothing to Report.

PRODUCTS:

Publications, conference papers, and presentations

Journal publications.

Mancuso P, Tricarico R, Bhattacharjee V, Cosentino L, Kadariya Y, Jelinek J, Nicolas E, Einarson M, Beeharry N, Devarajan K, Katz RA, Dorjsuren DG, Sun H, Simeonov A, Giordano A, Testa JR, Davidson G, Davidson I, Larue L, Sobol RW, Yen TJ, Bellacosa A. Thymine DNA glycosylase as a novel target for melanoma. *Oncogene* 38:3710-28, 2019. PMCID: PMC6563616.

Sannai M, Doneddu V, Giri V, Seeholzer S, Nicolas E, Yip S-C, Bassi MR, Mancuso P, Cortellino S, Cigliano A, Lurie R, Ding H, Chernoff J, Sobol RW, Yen TJ, Bagella L, and Bellacosa A. Modification of the base excision repair enzyme MBD4 by the small ubiquitin-like molecule SUMO1. *DNA Repair*, in press 2019.

Ross K, Chin K, Kim D, Marion C, Yen T, Bhattacharjee V. Methotrexate sensitizes drug resistant metastatic melanoma cells to BRAF V600E inhibitors dabrafenib and encorafenib. *Oncotarget* 9:13324-13336. 2018. PMCID: PMC5862581.

Books or other non-periodical, one-time publications. Nothing to Report.

Other publications, conference papers, and presentations. Nothing to Report.

Website(s) or other Internet site(s)

Nothing to Report.

Technologies or techniques

Nothing to Report.

Inventions, patent applications, and/or licenses

Nothing to Report.

Other Products

Nothing to Report.

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	***A. Bellacosa, M.D, Ph.D.***
Project Role:	Principal Investigator
Researcher Identifier (e.g. ORCID ID):	0000-0001-6278-6801
Nearest person month worked:	1
Contribution to Project:	Preparation and submission of internal IRB protocols and HRPO forms. Preparation and submission of internal IACUC protocols and ACURO forms. Design and implement TDG knockdown experiments and experiments with TDG inhibitors on lung cancer cell lines.
Funding Support:	N/A
Name:	***T. Yen, Ph.D.***
Project Role:	Principal Investigator
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	1
Contribution to Project:	Design and implement TDG knockdown experiments. Create inducible vector for shTDG. Assess viability of lung cancer cells after TDG knockdown by colony formation assay. Analyze candidate TDG inhibitors on lung cancer cell lines.
Funding Support:	N/A
Name:	***J. Hittle***
Project Role:	Technical Specialist
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	4
Contribution to Project:	Technical support such as tissue culture, produce biological reagents for the project, conduct TDG knockdown experiments and analyze lysates for knockdown. Performing clonogenic assay.
Funding Support:	N/A
Name:	***R. Prasad, Ph.D.***
Project Role:	Postdoctoral Associate
Researcher Identifier (e.g. ORCID ID):	

Nearest person month worked:	9
Contribution to Project:	Preparation of biological reagents for the project, including control and shRNA TDG lentiviruses. Tissue culture of lung cancer cell lines. Supply TDG inhibitors and test them on lung cancer cell lines. Analysis of DNA methylation patterns at centromeres and immunofluorescence for CENP-B.
Funding Support:	N/A

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Please see attached updated Other Support for key personnel. Changes are marked with a line in the right hand margin.

What other organizations were involved as partners?

Nothing to Report.

SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: Not applicable.

QUAD CHARTS: Not applicable.

APPENDICES: Not applicable.

Other Support

Bellacosa, Alfonso

Remaining salary support from institutional sources.

CURRENT

(PI: Yen) 6/1/2019 - 5/31/2020 5.0%
Sandy Rollman No Salary 0.60 calendar
Presence of a Genome-wide Hypomethylation Signature in Ovarian Cancer Patients Leads to a Novel Epigenetic Therapy for Precision Medicine
The major goal of this project is to validate the hypothesis that ovarian cancer may be treated by precision medicine.
Procuring Contracting/Grants Officer: Robin Cohen, 210 West Chester Pike, Suite 410, Havertown, PA 19083

W81XWH-17-1-0136 (PI: Bellacosa / Yen) 7/1/2017 - 6/30/2020 10.0%
DOD 1.20 calendar Thymine
DNA Glycosylase as a Novel Target for Lung Cancer (Multi-PI)
This grant is in a one year extension.
The major goals of this project are to: 1) Characterize the mechanisms by which TDG inhibition facilitates growth arrest, senescence, and cell death; and 2) Conduct translational studies of candidate TDG inhibitors as single agents, and in combination with existing chemotherapies and epigenetic therapies.
Procuring Contracting/Grants Officer: Amanda Carrera, 820 Chandler St., Fort Detrick, MD 21702

COMPLETED

4100077072

OVERLAP

None

Other Support

Yen, Timothy J.

Remaining salary support from institutional sources.

CURRENT

(PI: Yen) 6/1/2019 - 5/31/2020 5.0%
Sandy Rollman 0.60 calendar No SalarySignature in Ovarian
Presence of a Genome-wide Hypomethylation Cancer Patients
Leads to a Novel Epigenetic Therapy for Precision Medicine
The major goal of this project is to validate the hypothesis that ovarian cancer may be treated by precision medicine.
Procuring Contracting/Grants Officer: Robin Cohen, 210 West Chester Pike, Suite 410, Havertown, PA 19083

4100079728 (PI: Yen) 6/1/2018 - 5/31/2020 10.0%
PA DOH CURE 1.20 calendar
Identification of Tumor Suppressor Gene Targets in the Cancer Genome
The major goals of this project are to: 1) Identify and compare the target genes of Thymine DNA glycosylase (TDG) (factor A) in melanoma and pancreatic cancer; and 2) Develop Calling Card technology for BAP1 by fusing a transposase to and testing its expression in cells.
Procuring Contracting/Grants Officer: Penny Harris, Rm. 833 Health & Welfare Bldg., 625 Forster St., Harrisburg PA 17120

S10 OD023666 (PI: Yen) 9/1/2018 - 8/31/2019 NA
NIH (No Salary)
Multiphoton Microscopy System
This shared instrument grant application provides support for the purchase a multiphoton photon microscope (MPM) imaging system. Multiphoton microscopy provides users the ability: 1) for deep penetration imaging of cells in tissues of live animals and explants; 2) imaging tumor microenvironment (including stromal fibroblasts, infiltrating immune and inflammatory cells, blood and lymphatic vascular network, and the extracellular matrix) using SHG; 3) high-speed, low photobleaching real-time 3D imaging of model systems.
Procuring Contracting/Grants Officer: Karen Brummett, BD 1DEM, RM1042, 6701 Democracy Blvd., Bethesda, MD 20817

W81XWH-17-1-0136 (PI: Bellacosa / Yen) 7/1/2017 - 6/30/2020 10.0%
DOD 1.20 calendar Thymine
DNA Glycosylase as a Novel Target for Lung Cancer (Multi-PI)
This grant is in a one year extension.
The major goals of this project are to: 1) Characterize the mechanisms by which TDG inhibition facilitates growth arrest, senescence, and cell death; and 2) Conduct translational studies of candidate TDG inhibitors as single agents, and in combination with existing chemotherapies and epigenetic therapies.
Procuring Contracting/Grants Officer: Amanda Carrera, 820 Chandler St., Fort Detrick, MD 21702,

P30 CA006927 (PI: Fisher) 8/12/2016 - 7/31/2021 10.0%
NIH Salary only 1.20 calendar
Comprehensive Cancer Center Program at Fox Chase
The major goal of this Cancer Center Support Grant is to provide partial salary support for professional personnel, including senior and program leadership, administration, planning and evaluation, and

developmental funds, as well as support for 5 established peer-reviewed Research Programs, 12 Shared Research Resources and 2 Support Elements.
Procuring Contracting/Grants Officer: Candace Cofie, 9609 Medical Center Dr., Bethesda, MD 20892

OVERLAP

None