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TITLE: Biomarkers to Predict Repsonse to Multi-RTK Targeted Therapy in Advanced Colorectal Cancer

PRINCIPAL INVESTIGATOR: Bhuminder Singh

CONTRACTING ORGANIZATION: Vanderbilt University Medical Center

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13. SUPPLEMENTARY NOTES

14. ABSTRACT

Cetuximab is an antibody that targets the oncogene EGFR and is approved for use in advanced CRC. However, multiple resistance mechanisms to cetuximab exist that may be present at the outset or develop during treatment. We have shown a new mode of cetuximab resistance, due to increased phosphorylation of MET and RON RTKs. We also identified increased expression of the glutamate transporter, SLC1A7, in PDXs with high MET/RON phosphorylation. Here, we hypothesize that SLC1A7 contributes to the cetuximab resistance, and that modulation of glutamate levels may be a novel strategy to counter cetuximab resistance.

We will test this hypothesis by employing multiple CRC cell lines and patient-derived organoids (PDOs) with varying levels of SLC1A7 and their response to the EGFR-directed therapeutic, cetuximab. We will also genetically manipulate SLC1A7 in select lines (CRISPR-based knockout, or doxycycline-inducible overexpression) to test the cooperation of other genes like the glutamine transporter, ASCT2, or the mitochondrial glutamate transporter, SLC25A22. These experiments will also highlight the subcellular location or the pathways that cellular glutamate may be involved in CRC progression and drug resistance.

This year, we had proposed to generate SLC1A7 overexpression and knockout / knockdown constructs in multiple CRC lines and PDOs. We have been able to generate the cloning strategy and have infected the lines with several lentiviral particles (CRISPR-knockout and doxycline-inducible overexpression. We will next test individual clones for their desired (loss or increased) expression of SLC1A7 and then will test contribution to CRC progression and cetuximab resistance.

15. SUBJECT TERMS

NONE LISTED

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRDC
Unclassified	Unclassified	Unclassified	Unclassified	14	19b. TELEPHONE NUMBER *(include area code)*

TABLE OF CONTENTS

1. **INTRODUCTION:**

1. INTRODUCTION. Cetuximab, an antibody that targets EGFR is approved for use in *KRAS* wild-type advanced CRC. However, multiple resistance mechanisms to cetuximab exist that may be present at the outset or develop during treatment. *We have shown a new mode of cetuximab resistance, due to increased phosphorylation of MET and RON RTKs.* We also identified increased expression of the glutamate transporter, SLC1A7, in PDXs with high MET/RON phosphorylation. Here, we hypothesize that SLC1A7 contributes to the cetuximab resistance, and that modulation of glutamate levels may be a novel strategy to counter cetuximab resistance.

2. **KEYWORDS:**

2. KEYWORDS. Colorectal cancer (CRC), EGFR (Epidermal Growth Factor Receptor), cetuximab, cetuximab resistance, drug resistance, glutamine/glutamate metabolism, patient-derived xenografts (PDXs), patient-derived organoids (PDOs)

3. **ACCOMPLISHMENTS:**

3. ACCOMPLISHMENTS (MAJOR GOALS). The Specific Aims of project and overall goals remain unchanged from those originally outlined in the final approved Statement of Work. Major goals of this project are: **1)** Testing the role of glutamate metabolism (via SLC1A7 uptake) on colorectal cancer progression and response to the EGFR-directed therapeutic, cetuximab; **2)** testing the effect of related cellular processes including glutamine uptake (via ASCT2), mitochondrial glutamine to glutamate conversion (by GLS1/2), and mitochondrial glutamate uptake (via SLC25A22) on glutamate metabolism and cetuximab resistance. The long-term goal of this project is to determine if modulating the glutamate metabolism can help prevent, delay, or overcome cetuximab resistance in colorectal cancer.

- **What was accomplished under these goals?**
  - *For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.*

3. ACCOMPLISHMENTS (ACTIVITIES, OBJECTIVES, RESULTS & OUTCOMES).

Major activities: During this period, mostly the conceiving of tools required, and generation of resources was proposed, including selection of CRC lines and organoids, CRISPR-mediated knockout of genes of interest and overexpression of genes of interest.

Specific objectives: The generation of tools and resources proposed to be generated will be needed to test the contribution of individual genes to the cetuximab resistance phenotype and whether they may be novel therapeutic targets.

Significant results and key outcomes: In first year of the award, we had outlined to focus our efforts to complete Subtasks 3.1, 3.2, and 3.3.

Within **Subtask 3.1** we had proposed to generate CRC cell lines and organoids with SLC1A7 knockdown in SC, CC-CR or ~3 PDOs with high pMET/pRON levels. We have accomplished the following in this subtask.

- We have designed CRISPR knockout lentiviral constructs and the plasmid outlined below in Fig. 1.

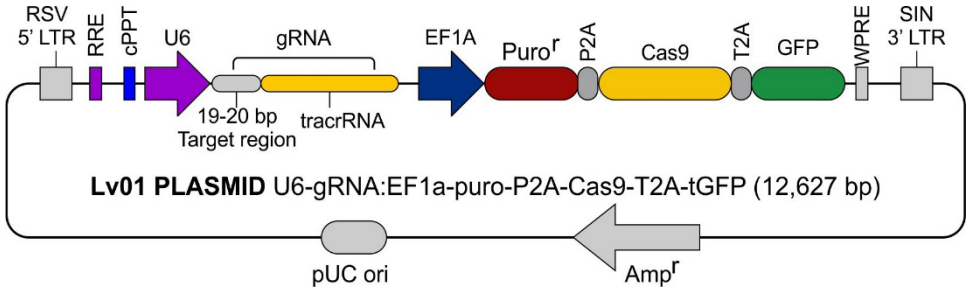

Fig. 1. Outline of CRISPR knockout lentiviral plasmid. Abbreviations: RSV LTR = Rous sarcoma virus – long terminal repeat; RRE = Rev response element; cPPT = central polypurine tract; gRNA = guide RNA; EF1A = The EF-1α promoter; Puro^r = puromycin resistance marker; P2A, T2A = inserts for ribosomal "skipping" during translation to generate three individual proteins; WPRE = Woodchuck Hepatitis Virus Posttranscriptional Regulatory Element; SIN 3' LTR = U3 deletion in 3'LTR - self-inactivation.

- We have infected the CRC cell line, DiFi, with the CRISPR knockout lentiviral particles and selected and FACS-sorted the lines to generate enriched pools and isolate individual clones. More than 20 clones were generated that were positive by fluorescent marker, which confirms incorporation of the transgenic genomic material (Fig. 2). We will next perform western blot analysis of these clones to confirm removal of SLC1A7 protein expression.

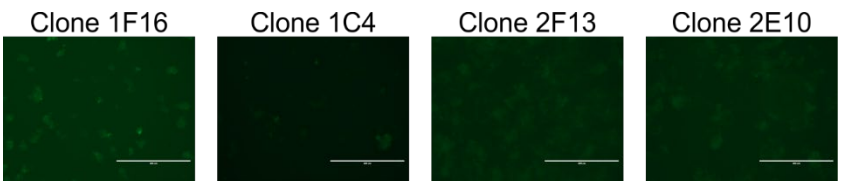

Fig. 2. Selecting for SLC1A7 in CRC cell line, DiFi. Representative four DiFi clones that were FACS sorted for the GFP after infecting them with the SLC1A7 lentiviral particles (from Fig. 1). Scale bars: 400 μm. Clone 1F16 seems to have higher GFP expression.

Continued on next page…

3. ACCOMPLISHMENTS (ACTIVITIES, OBJECTIVES, RESULTS & OUTCOMES). Contd…

Within **Subtask 3.2**, we had proposed to generate CRC cell lines and organoids with SLC1A7 overexpression in lines with low SLC1A7 expression. The cell lines were chosen based on SLC1A7 expression from the Cancer Cell Line Encyclopedia (CCLE) from Broad Institute.

- We have designed SLC1A7 tagged with HaloTag at its amino-terminus with a two-plasmid system for doxycycline inducible overexpression of SLC1A7 (Fig. 3). Tag position has been chosen after bioinformatic analysis to preserve the membrane topology.

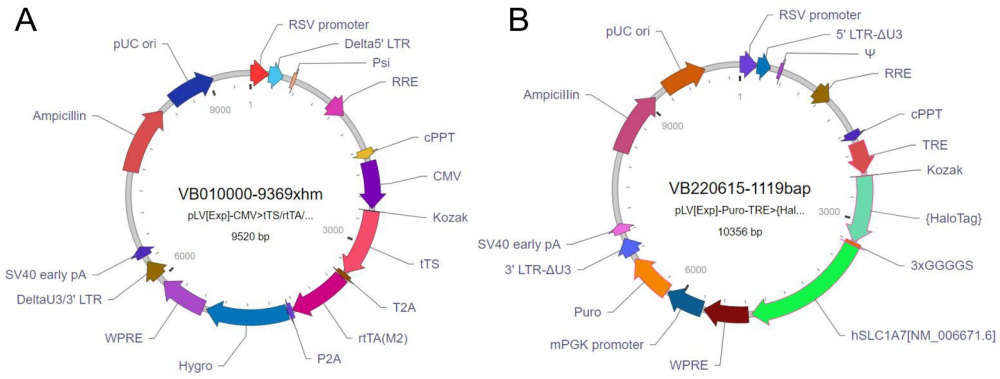

Fig. 3. Doxycline-induced expression of SLC1A7 (two-plasmid system). A) Lentiviral plasmid #1 to express rTS and rtTA with Hygromycin selection. B) Lentiviral plasmid with human SLC1A7 incorporated along with HaloTag at its N-terminus. Detailed maps of plasmids and nucleotide sequences may be found here: A) https://en.vectorbuilder.com/vector/VB010000-9369xhm.html B) https://en.vectorbuilder.com/vector/VB220615-1119bap.html

- We have infected four CRC cell lines with low SLC1A7 expression with the lentiviral particles containing doxycycline-inducible Halo-tagged human SLC1A7 constructs. These lines have been co-infected with the tTS/rtTA expression plasmid (needed for dox induction) and the infected lines are now under selection. We anticipate generating overexpressing pools and clones in 1-2 months and then perform related experiments.

In **Subtask 3.3** we had proposed testing cetuximab resistance in lines generated in Subtasks 3.1 and 3.2. This task has not been completed as we are waiting on reagents being generated and characterized from Subtasks 3.1 and 3.2. We anticipate completing this subtask within 3-6 months after resource generation.

Discussion of stated goals not met: As mentioned above and elsewhere, combined shortage of personnel and usual technical difficulties.

- **What opportunities for training and professional development has the project provided?**
  - *If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state "Nothing to Report."*
  - *Describe opportunities for training and professional development provided to anyone who worked on the project or anyone who was involved in the activities supported by the project. "Training" activities are those in which individuals with advanced professional skills and experience assist others in attaining greater proficiency. Training activities may include, for example, courses or one-on-one work with a mentor. "Professional development" activities result in increased knowledge or skill in one's area of expertise and may include workshops,*

conferences, seminars, study groups, and individual study. Include participation in conferences, workshops, and seminars not listed under major activities.

A Graduate Student, Vivian Truong Jones, is being trained during the process of completion of these projects.

- **How were the results disseminated to communities of interest?**
  - *If there is nothing significant to report during this reporting period, state "Nothing to Report."*
  - *Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of these project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities.*

Nothing to report.

- **What do you plan to do during the next reporting period to accomplish the goals?**
  - *If this is the final report, state "Nothing to Report."*
  - *Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives*

We plan to accomplish completion of SLC1A7 CRISPR knockout and then test the contribution of SLC1A7 expression to cetuximab resistance.

Alternate strategy: If CRISPR is not achieved to desired satisfaction, a shRNA-based SLC1A7 knockdown will be achieved in the lines of interest.

We also plan to test the effect of SLC1A7 inhibitor on cetuximab resistance by itself or in combination with the inhibition of glutamine transporter, or mitochondrial glutaminase (GLS1/2).

In the reverse experiment, we plan to generate inducible overexpression of cell-surface and mitochondrial transporters to test their potential cooperative action to enhance glutamate metabolism and contributing to cetuximab resistance.

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4. **IMPACT:**

- **What was the impact on the development of the principal discipline(s) of the project?**
  - *If there is nothing significant to report during this reporting period, state "Nothing to Report."*
  - *Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).*

Nothing to report.

- **What was the impact on other disciplines?**
  - *If there is nothing significant to report during this reporting period, state "Nothing to Report."*
  - *Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.*

Nothing to report.

- **What was the impact on technology transfer?**
  - *If there is nothing significant to report during this reporting period, state "Nothing to Report."*
  - *Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:*
    - *transfer of results to entities in government or industry;*
    - *instances where the research has led to the initiation of a start-up company; or*
    - *adoption of new practices.*

Nothing to report.

- **What was the impact on society beyond science and technology?**
  - *If there is nothing significant to report during this reporting period, state "Nothing to Report."*
  - *Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:*
    - *improving public knowledge, attitudes, skills, and abilities;*
    - *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*
    - *improving social, economic, civic, or environmental conditions.*

Nothing to report.

5. **CHANGES/PROBLEMS:** *The Project Director/Principal Investigator (PD/PI) is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer whenever*

there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:

- **Changes in approach and reasons for change**
  - *Describe any changes in approach during the reporting period and reasons for these changes. Remember that significant changes in objectives and scope require prior approval of the agency.*

Nothing to report.

- **Actual or anticipated problems or delays and actions or plans to resolve them**
  - *Describe problems or delays encountered during the reporting period and actions or plans to resolve them.*

Hiring delays: This year's activities continue to be hampered due to COVID-19. Hiring of Research fellows has been difficult, in part, due to travel restrictions. However, to cope with these limitations, interviews of all candidates were conducted remotely. For this project, we have offered one candidate a Research Instructor position. The candidate is facing additional delays in securing limited VISA interview spots at the US Embassy, but we anticipate him joining later this year. Meanwhile, a PhD student has been helping to move the project forward. Since the student is supported by a training grant, she is not drawing salary from this grant.

- **Changes that had a significant impact on expenditures**
  - *Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.*

Hiring delays: As mentioned above, hiring delays have led to *significant less spending* on the project. However, the project is expected to resume and recover as hiring for a postdoc fellow has been finalized and the person has applied for USA VISA to enter the country. Additionally, the graduate student will continue to work on the project. *We thus request that the unused funds from year 1 be made available in the subsequent years of this project to bring the project to successful completion.*

- **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**
  - *Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.*

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.

6. **PRODUCTS:** *List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."*

    - **Publications, conference papers, and presentations**
      Report only the major publication(s) resulting from the work under this award.

        - **Journal publications.** *List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume: year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

Nothing to report.

        - **Books or other non-periodical, one-time publications.** *Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: Author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

Nothing to report.

        - **Other publications, conference papers, and presentations.** *Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.*

Nothing to report.

- **Website(s) or other Internet site(s)**
  List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

Nothing to report.

- **Technologies or techniques**
  Identify technologies or techniques that resulted from the research activities. In addition to a description of the technologies or techniques, describe how they will be shared.

Nothing to report.

- **Inventions, patent applications, and/or licenses**
  Identify inventions, patent applications with date, and/or licenses that have resulted from the research. State whether an application is provisional or non-provisional and indicate the application number. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.

Nothing to report.

- **Other Products**
  Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment, and/or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:

  - *data or databases;*
  - *biospecimen collections;*
  - *audio or video products;*
  - *software;*
  - *models;*
  - *educational aids or curricula;*
  - *instruments or equipment;*
  - *research material (e.g., Germplasm; cell lines, DNA probes, animal models);*

- *clinical interventions;*
- *new business creation; and*
- *other.*

Nothing to report.

7. **PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS**

- **What individuals have worked on the project?**

  - *Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change."*

    ***Example**:*

Name:	*Mary Smith*
Project Role:	*Graduate Student*
Researcher Identifier (e.g. ORCID ID):	*1234567*
Nearest person month worked:	*5*
Contribution to Project:	*Ms. Smith has performed work in the area of combined error-control and constrained coding.*
Funding Support:	*The Ford Foundation (Complete only if the funding support is provided from other than this award).*

Unchanged contributors: *Bhuminder Singh, Galina Bogatcheva, Peter J Dempsey, Gregory Dan Ayers*

New contributor Name: *Vivian Truong Jones, PharmD*
Project Role: *Graduate Student*
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: *6*

Contribution to Project: *Dr. Jones has performed analysis of CRC cell lines for the RNA expression of key proteins including SLC1A7 and SLC25A22. Additionally, Dr. Jones has designed the CRISPR knockout and inducible SLC1A7 overexpression constructs and performed lentiviral infection of CRC lines, and subsequent antibiotic selection and cloning. Moreover, Dr. Jones has isolated DNA and RNA from select CRC patient-derived organoids to determine their genetic background and expression levels of proteins of interest.*

Funding Support: *Training grant (T32 GM07628, PI: Joey Barnett)*

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

    - *If there is nothing significant to report during this reporting period, state "Nothing to Report."*

    - *If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.*

Nothing to report.

- **What other organizations were involved as partners?**

    - *If there is nothing significant to report during this reporting period, state "Nothing to Report."*

    - *Describe partner organizations - academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) - that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed.*
      Provide the following information for each partnership:

        - **Organization Name:**

        - **Location of Organization:** *(if foreign location list country)*

        - **Partner's contribution to the project** *(identify one or more)*

            - **Financial support;**

            - **In-kind support** *(e.g., partner makes software, computers, equipment, etc., available to project staff)*;

            - **Facilities** *(e.g., project staff use the partner's facilities for project activities)*;

            - **Collaboration** *(e.g., partner's staff work with project staff on the project)*;

            - **Personnel exchanges** *(e.g., project staff and/or partner's staff use each other's facilities, work at each other's site)*; and

            - **Other.**

Nothing to report.

8. **SPECIAL REPORTING REQUIREMENTS**

- **COLLABORATIVE AWARDS:** *For collaborative awards, independent reports are required from **BOTH** the Initiating PI and the Collaborating/Partnering PI. A duplicative report is acceptable;*

however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to https://ebrap.org for each unique award.

- **QUAD CHARTS:** *If applicable, the Quad Chart (available on https://www.usamraa.army.mil) should be updated and submitted with attachments.*

9. **APPENDICES:** *Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc. Reminder: Pages shall be consecutively numbered throughout the report.* ***DO NOT RENUMBER PAGES IN THE APPENDICES.***