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TITLE: Endogenous Retrovirus Expression, Chromatin Abnormalities and Response to Immune Checkpoint Blockade in Clear Cell Renal Cell Cancer

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14. ABSTRACT

In this study, we investigate the role of re-expression of endogenous retroviruses (ERV) as an alternative mechanism of immune activation that may be relevant in ccRCC. Our preliminary data demonstrate that ERVs, including ERV3.2 are highly expressed in a subset of ccRCC and this expression is associated with evidence of CD8+ T-cell infiltration and with response to single agent immune checkpoint blockade. We have found that a subset of ccRCC have increased expression of specific immune checkpoints including LAG3, suggesting that novel LAG3 antibodies may be active in ccRCC. Treatment of ccRCC cell lines and models with epigenetic modifying agents can increase ERV expression. Analyses is ongoing in both cell models and tissue specimens to further investigate how ERV expression is associated with both chromatin changes and immune activation in renal cancer. Our overall aim is to investigate the mechanisms of dysregulated ERV expression in a subset of ccRCC and could function and its potential role as a biomarker of response to ICB

15. SUBJECT TERMS

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1. INTRODUCTION

Clear-cell renal-cell carcinoma (ccRCC) has significant response rates to single immune checkpoint blockade (ICB), despite having no known markers of response such as either high mutation burden or evidence of exogenous viral infection. In this study, we investigate an alternative mechanism of immune activation that may be relevant in low mutation burden cancers such as ccRCC: re-expression of repetitive RNA elements including endogenous retroviruses (ERV). Intriguinly, ccRCC has a high incidence of mutations in chromatin modifying genes, including PBRM1, SETD2, KDM5C, and BAP1. We hypothesize that chromatin and epigenetic abnormalities present in a subset of ccRCC may lead to expression of normally silenced repetitive elements, such as ERV, leading to innate immune activation and response to ICB. We are investigating this hypothesis through analyses of both cell line models of ccRCC and human tumor samples. If successful this project will lead to new insight into a novel mechanism of immunogenicity, and lead to development of a tractable biomarker of response to immune checkpoint therapy that may be independent of mutation burden.

2. KEYWORDS

Renal Cell Carcinoma, Endogenous Retroviruses, chromatin, immune checkpoint therapy, epigenetics

3. ACCOMPLISHMENTS:

3.1 What were the major goals of the project?

Specific Aim 1: Determine the relationship between ERV expression and perturbations in DNA methylation, histone modification and mutations in chromatin remodeling genes in ccRCC.

Major Task 1: Analysis of Methylation and ERV expression in ccRCC :

Milestones

1. (months 1-3) Local IRB and regulatory approvals for tissue analysis:

Status at Month 24: : IRB /regulatory approvals obtained for tissue analysis.

2. (months 1-18) Obtain archived kidney cancer specimens and determine if patterns of focal and global DNA methylation are associated with abnormal expression of ERVs.

Status at month 24: All archived kidney cancers specimens are now available and currently undergoing analysis.

Major Task 2: Analsyis of Histone Modifications in ccRCC:

Milestone (Months 12-24)

1.Determine if landscape of histone modifications as analyzed by IF or by ChIP correlates with DNA methylation at ERV loci and ERV expression in ccRCC

Status at month 24: Specimens have been identified and we awaiting ERV analysis results in order to move ahead with IF and CHIP in a subset of specimens.

Major Task 3: Determine if DNMT inhibitors and epigenetic modifiers can induce ERV expression in ccRCC cell lines

Milestone :Determine if ERV expression in ccRCC cell lines can be manipulated by treatment with epigenetic modifying agents.

Status at month 24: We have demonstrated that DNA demethylating agents can potently induce ERV expression in a set of ERV cell lines and cell models. We are now working on testing the effect of EHMT1/EHMT2 histone methyl transferase inhibitors in the same systems with preliminary data also showing induction of innate immune pathways and ERV expression.

Specific Aim 2. Determine relationship between ERV expression and the immune microenvironment in ccRCC

Major Task 1: Analysis of Multiparameter IF

Milestone (month 6-18): Determine if ERV expression correlates with presence of specific immune cells subsets in the tumor microenvironment.

Status: We now have a full set of specimens; a subset of samples have been analyzed for immune cell subsets using multiple assays. Preliminary experiments show feasibility of multiparameter IF looking at immune subsets. Analysis of ERV expression and correlation of immune populations with ERV status are underway.

Major Task 2: Analysis of immune gene expression by NanoString

Milestone (months 6-18) : Determine if ERV expression in ccRCC is associated with RNA evidence of activation of specific immune pathways

Status at month 12.: Specimens obtained and a subset have been analyzed for immune pathway activation. Further analysis of ERV expression and correlation of immune pathways activation with ERV status are underway.

Major Task 3: Flow-cytometry analysis of tumor microenvironment

Milestone (Months 12-24): Determine if ERV expression is associated with presence of specific immune populations in the tumor microenvironment by flow cytometry.

Status at month 24. : Fresh specimens have been identified, and flow cytometry analysis is being planned for the coming year.

Specific Aim 3: Examine the relationship between ERV3.2 and response to immune checkpoint blockade in ccRCC.

Major Task 1 : Analysis of ERV expression with response to immune checkpoint blockade

Milestone (months 6-24): determine if ERV expression in FFPE specimens of ccRCC is associated with clinical response to immune checkpoint blockade.

Status at month 24: : IRB approval for non-interventional study was obtained at all sites; enrollment has begun. Specimens for analysis have been identified and analysis for ERV expression is now underway.

Major Task 2: Analysis of acquired resistance to immune checkpoint therapy

Milestones: (Months 6-24): Determine if progression from immune checkpoint therapy in ccRCC is associated with repression of ERV expression.

Status as month 24: We have identified patients with progression to ICB at both sites; and are continuing to identify and obtain appropriate samples for analysis.

3.2 What was accomplished under these goals?

Specific Aim 1: Determine the relationship between ERV expression and perturbations in DNA methylation, histone modification and mutations in chromatin remodeling genes in ccRCC.

Activities: Final IRB approvals across all sites have been obtained. Frozen specimens are now available for analysis of histone modifications and DNA methylation and correlation with ERV expression by RT-PCR. Bioinformatic analysis has nominated several other ERVS as being of potential interest in ccRCC in addition to ERV3.2. RT-PCR based assays have been developed for these and validated using cell lines s and are now ready to be employed on primary cancer samples. We have also completed analysis of a set of ccRCC cell ines as outlined in prior technical report, and now have extended to other cell models. We are also examining the effect of histone methyl transferase inhibitors, including EHMT1/2 inhibitor , both alone and in combination with DNA methylation inhibitors (decitabine), on ERV expression and induction of interferon response pathway in these models.

Results: The effect of increasing doses of the DNMT-inhibitor decitabine on expression of a set of specific ERVs and other repetitive RNA species was evaluated by RNA sequencing in normal human kidney cell line (HCK) and in ccRCC cell line 786O. As presented in last report, decitabine treatment led to significant increase in a set of ERVs including ERV3.2 in 786-O cell with a much greater effect in the ccRCC cell line compared with the normal kidney cell line. This work has been published. We are now in process of extending this work to look at effect of other epigenetic manipulations including use of UNC0638, a inhibitor of the histone methyltransferases EHMT1 and EHTM2. Preliminary data show that UNC0638 treatment can potently induce STING in cell lines; its effect on ERV expression in the ccRCC models are now underway.

Specific Aim 2: Determine relationship between ERV expression and the immune microenvironment in ccRCC.

Activities: Although we had some unexpected regulatory and other delays, we now have IRB approved protocols for he project across all sites and we have now identified and retrieved high quality specimens of ccRCC and associated normal tissue CINJ tissue repository. We were able to perform some proof of concept studies on archived specimens to show ability to perform multi-IF with ability to look a expression of different markers, including PD-1, PD-L1 and cytokeratin in ccRCC samples. We anticipate moving forward with these assays on the full set of specimens over the next NCE period.

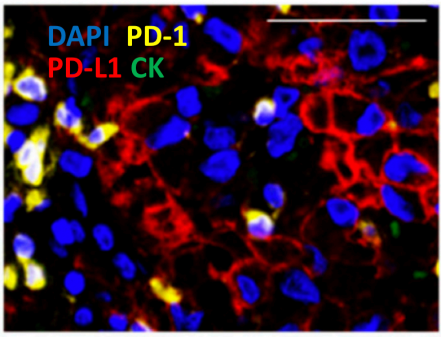

Figure 1. Representative multiplex IF staining of ccRCC specimen, stained for PD-1, PD-L1 and CK7

Results: As outlined in the prior technical report, we were able to perform some analyses on public data sets that identified LAG3 as a potent marker of immune activation in a subset of ccRCC, and this may function as a better marker of immunogenicity than PD-L1. These results were published last year (Panda et al, Oncoimmunology , 2020). We are now in the process of looking at correlation of LAG3 with ERV expression and other innate immune markers in a set of samples from CINJ. These results raise the exciting and unanticipated possibility that LAG3 inhibitors may be particularly effective in ERV+ ccRCC. We also have performed initial IF experiments that show proof of concept that we can look at immune markers including PD-1, PD-L1 in clinical specimens (Figure 1).

Specific Aim 3: Examine the relationship between expression of ERV3.2 and response to immune checkpoint blockade in ccRCC.

Activities: Again despite some delays, we now have IRB approvals for enrollment and specimen acquisition at all sites and we have begun enrolling patients with ccRCC who have been treated with immune checkpoint therapy. Analysis of prior archived specimens are underway, with preliminary data showing ability to obtain high quality RNA sequencing data and immune cell subset data from these FFPE specimens . We anticipate being able to process specimens from these samples over the next (NCE)period.

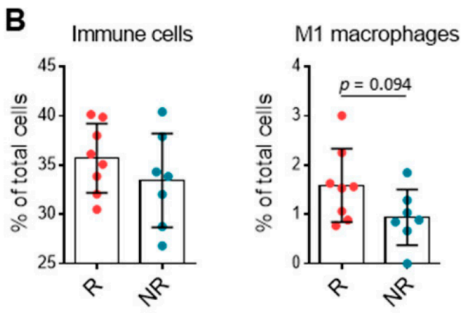

Figure 2: Deconvolution of total immune cell proportion and proportion of MI macrophages from RNA sequencing data obtaind from ccRCC specimens of responders(R) vs non-responders (NR) to immune checkpoint therapy.

Results: Analysis of pre-existing specimens was done as proof of concept to show we can get high quality RNA sequencing data from archived specimens and these can be used to identify lymphocyte subsets. Initial data from pre-existing samples(Figure 2) show that total lymphocytes and are not significantly different between responder and non-responders to immune checkpoint therapy. This suggests that specific lymphocyte subsets may be more informative than total lymphocyte populations and this analysis is now underway. MI macrophages showed a trend to be enriched in responder; we will interrogate this possibility in greater detail in the cohort being analyzed in this aim. We have also worked to refine and validate ERV RT-PCR assay using positive and negative control cell line specimens. These assays are now ready to be deployed on the tissue specimens.

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

Nothing to report.

3.4 How were the results disseminated to communities of interest?

Nothing to report.

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

Over the next reporting period (approved no-cost extension) we plan to complete the experimental plan outlined in the SOW. This includes:

1. Complete the analysis of histone modification and DNA methylation landscape in the archived samples of ccRCC that have now underway
2. Further analyze effect of epigenetic modifying treatments, including histone methyltransferase inhibitors on ERV expression in ccRCC cell lines and cell models.
3. Complete analysis of immune microenvironment in ccRCC specimens using multiparameter IF and RNA analyses and correlate these with correlation with ERV expression status.
4. Complete analysis of tissue specimens of patients with ccRCC who are or have been treated with immune checkpoint therapy to observational protocol at both sites (Rutgers and VMC) and correlate ERV expression, and immune markers with response to ICB
5. Present findings at major cancer meetings and prepare manuscripts for publication.

4. IMPACT

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

Nothing to report

4.2: What was the impact on other disciplines? Nothing to report

4.3: What was the impact on technology transfer? Nothing to report

4.4: What was the impact on society beyond science and technology? Nothing to report

5. CHANGES/PROBLEMS

5.1: Changes in approach and reasons for change

Nothing to report

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

The continuing COVID pandemic has led to continued delays in approval and initiation of IRB approved protocols. Importantly, during COVID crisis enrollment to non-therapeutic trials was halted, leading to delays in tissue acquisition for parts of Aims 1 and 2, and delay in activation of the observational protocol outlined in Aim 3, delays in execution of subcontracts to Vanderbilt. However, these are now all in place and both laboratory and other staff are in place to execute the project over the next, NCE, period. Proof of concept studies have shown feasibility of planned analysis and we are excited to implement them .

5.3: Changes that had a significant impact on expenditures

Disruption from the COVID pandemic led to delays in both the regulatory process and obtaining Vanderbilt subcontracts leading to delays in access too new samples for analyses, and we could not perform all of the tissue analyses we had budgeted for years 1 and 2. . This has led to a significant unobligated balance , for which we have applied for and been approved for a no-cost extension (Carry over of approximately).

We anticipate that we will be able to now complete processing and analysis of samples over the NCE period.

5.4: Significant changes in use of human subjects:

Nothing to report

5.5 Significant changes in use or care of vertebrate animals

Nothing to report

5.6 Significant changes in use of biohazards and/or select agents

Nothing to report

6. PRODUCTS

6.1 Publications:

1. Aguirre A. de Cubas, William Dunker, Andrew Zaninovich, Rachel A. Hongo, Anuj Bhatia, Anshuman Panda, Kathryn E. Beckermann, Gyan Bhanot, Shridar Ganesan, John Karijolich, and W. Kimryn Rathmell. DNA hypomethylation promotes transposable element expression and activation of immune signaling in renal cell cancer, JCI-Insight: 2020;5(11):e137569. https://doi.org/10.1172/jci.insight.137569. Published.

2. E Shiuan, A Reddy, S Dudzinski, A Lim1, A Sugiura, R Hongo, K Young, XD Liu, C Smith, J O'Neal, K Dahlman, R McAlister, B Chen, K Ruma, Nathan Rosco, Johovana Bender, Joolz Ward, Ju Young Kim, C Vaupel, J Bordeaux, S Ganesan, BG Vincent, NB Davis, SM Haake, JC Rathmell, E Jonasch, BI Rini, WK Rathmell, and KE Beckermann. Clinical correlates and multiplatform molecular analysis of response to anti-PD-1/PD-L1 in renal cell carcinoma. Cancers (Basel). 2021 Mar 23;13(6):1475. doi: 10.3390/cancers13061475.

6.2 Presentations

Nothing to report.

6.3 Websites

Nothing to report.

6.4 Technologies or techniques

Nothing to report.

6.5 Inventions, Patent Applications and/or licences.

Nothing to report.

6.6 Other Products

Nothing to report.

7. PARTICIPANTS AND OTHER COLLABORATING ORGANIZATIONS 7.1 What individuals have worked on this project:

Name	Shridar Ganesan
Project Role	PI
Researcher ID	0000-0002-5404-3044
Person Months Worked	1.2
Contribution	As PI, contributed to obtaining regulatory approvals , oversight of all experiments and analyses,

Name	Gyan Bhanot
Project Role	Co-Investigator
Researcher ID	
Person Months Worked	1.2
Contribution	Guide bioinformatic analysis

Name	Anshuman Panda
Project Role	Post-Doctoral fellow
Researcher ID	
Person Months Worked	8.4
Contribution	Implements and performs bioinformatic analyses, RNA sequencing

Name	Ming Yao
Project Role	Research Assistant
Researcher ID	
Person Months Worked	0.6
Contribution	Helped with sample processing and lab experiments

Name	Katy Beckerman
Project Role	Co-Investigator
Researcher ID	
Person Months Worked	1.2
Contribution	Contributes to procurement and analysis of clinical specimens and cell data

Name	Rachel Hongo
Project Role	Research Assistant
Researcher ID	
Person Months Worked	2.4
Contribution	Prepares cell lines and specimens for genomic and other analyses; assistass in data analysis

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

Shridar Ganesan: The PI now has a new funded project on an NCI PO1 project
PO1 830210/830205. 04/01/2021-03/31/2026 3.0 Calendar Months
PI: Shen/.Ganesan, NIH/NCI
Role: PI project 3
Annual Direct Cost:
Chromatin Modifiers oof the BRCA-related DNA repair pathways
Major Goals: To determine the roles of chromatin-modifying enzymes G9a/GLP on modulating BRCA-related DNA repair pathways and DNA repair choice.

Overlap: NONE

Shridar Ganesan : Is now a coinvestigator Co-investigator for a project funded by DoD BCRP 830378 (PI: Liotta):
BCRP 830378 04/01/2021-03/31/2024. 0.3 Calendar months
PI: Liotta, DoD
Role: Co-investigator
Total Cost:
Computational approaches for identifying epigenomic contexts of somatic mutations.
Major Goals: This proposal aims to use phosphoproteomics to analyze HER2 pathway activation in breast cancer

Overlap: NONE

Shridar Ganesan : Is now a co-investigator Co-investigator for a NCI RO1 (PI: Mitrofanova).
R01LM013236 09/09/2020-08/31/2024 0.3 Calendar months
PI: Mitrofanova
Role: Co-investigator
Annual Direcd Cost
Application of Statistical and Machine Learning to Predictive Modeling of Treatment Response.
Major Goals: We propose to build an open-source scalable generalizable method that would assist experimentalists and clinicians on assessing patient's risk of developing therapy resistance and build a platform for patient-centric clinical decision/

Overlap: NONE

Shridar Ganesan : Is now a coinvestigator Co-investigator for a ACS/Pfizer grant (PI: Kinney)
ACS 11/18/20-12/31/22 0.30 Calendar Months
PI: Kinney
Role: Co-investigator
Total Cost: 400,000
Reducing Racial Disparities in Cancer Care with PINPOINT-127870
Major Goals: Understand racial disparities in use of tumor sequencing, targeted therapies and immunotherapy
Overlap: None

Shridar Ganesan's effort on BCRF grant has ended
Shridar Ganesan's effort on NIH/NCI grant R01CA202752 has ended.
Shridar Ganesan's effort on NIH/NCI grant 5UM1CA186716 has ended.

Katy Beckerman is now a co-investigator for a Kidney Cancer Clinical Trials Consortium grant from DoD, CDMRP (PI: Brian Rini).
DoD/CDMRP/KCRP 09/15/2021 - 09/14/2023 1.8 calendar months
PI: Rini
Role: Co-investigator
Total Cost:
Kidney Cancer Clinical Trials Consortium
Major Goal: The goal of the Vanderbilt-Ingram Cancer Center's (VICC) participation in the Kidney Cancer Research Consortium (KCRC) is to meaningfully contribute to Phase I and II clinical research in the context of this multi-institutional collaboration.

Overlap: NONE

Katy Beckerman's effort on K12CA090625-21 effort ended

7.3. **What other organizations were involved as partners?**

Nothing to report.

8. SPECIAL REPORTING REQUIREMENTS

Nothing to report

9. Appendices: None