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14. ABSTRACT

LncRNAs CRNDE is one of the most significantly elevated-expressed genes in HCC. CRNDE levels are reversely correlated with HCC patients' prognosis. Based on our previous data, we assumed that HCC-derived CRNDE acts as inhibitory messenger molecular to impair host immunoreaction in the tumor microenvironment, and thus causing immune evasion and promoting HCC progression. During the 1st year of the performance, we evaluated the impact of CRNDE on tumor growth *in vivo* using a novel mouse model of HCC induced by HDTV (Hydrodynamic tail vein) injection of transposase (sleeping beauty)-based plasmids expressing Yap^{S127A} and $\beta\text{-catenin}^{\Delta N90}$. we observed that co-injecting CRNDE-expressing plasmids with $Yap^{S127A}/\beta\text{-catenin}^{\Delta N90}$ promotes HCC growth; while co-injecting shCRNDE plasmids with $Yap^{S127A}/\beta\text{-catenin}^{\Delta N90}$ attenuate Yap/β-catenin-induced HCC growth. Further RNA-seq analyses of these mouse HCC tissues indicate that immune system activation level was greatly elevated in CRNDE-depleted HCC when compared with control HCC, demonstrating as higher expression of immune-response related genes; more infiltrating CD8 T-cells, CD4 memory T-cells and NK cells in tumor; and higher level of CD8 T-cells activation in tumor tissues. Next, in order to determine the effect of CRNDE on TILs functions, we carried out granzyme B ELISPOT assays using mouse TILs, which were isolated from mouse HCCs induced by HDTV injection of $Yap^{S127A}/\beta\text{-catenin}^{\Delta N90}$ in combination with CRNDE over-expression or shCRNDE plasmids and their respective control plasmids. Our data demonstrated that HCCs with CRNDE over-expression/depletion have much less/more granzyme B-secreting TILs than their corresponding control HCCs respectively. These results further support our initial assumption

15. SUBJECT TERMS					
HCC, LncRNA, CRNDE, Immunosuppression, Tumor Infiltrating Lymphocyte					
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1. Introduction

Tumor-infiltrating lymphocytes (TILs) in HCC tissues are functionally compromised, impeding anti-tumor immunity. While inhibitory receptor-ligand pathways partly attribute to compromised TIL response in HCC, additional mechanisms likely also exist in the HCC microenvironment. Our preliminary results indicate that long non-coding RNA CRNDE can be transported from HCC cell into TIL, and the transferred HCC-derived CRNDE acts as a crucial factor to inhibit TIL anti-tumor functions in the HCC microenvironment. The objective of this projects is to assess the potential of CRNDE as a novel therapeutic target for effective anti-HCC immuno-therapy. In this project, we plan to explore the functional impact of CRNDE on hepatic carcinogenesis through regulating TILs activation; we also plan to determine the mechanism by which HCC-derived CRNDE inhibits TIL functions; as well as to evaluate the potential therapeutic effects of CRNDE specific gapmer-ASO (next-generation anti-sense oligonucleotides) in HCC.

2. Keywords

Hepatocellular carcinoma (HCC)
Long non-coding RNA (lncRNA)
Colorectal Neoplasia Differentially Expressed (CRNDE)
Immunosuppression
Tumor Infiltrating Lymphocyte (TIL)
Exosome
Hydrodynamic injection

3. Accomplishments

- Major goals of the project (anticipated date of completion, actual completion date)

  First year (9/15/2019 to 9/15/2020):

  1. To obtain ACURO approval (12/15/2019, 1/16/2020)
  2. To determine the effect of CRNDE on TIL functions (2/15/2020, 2/10/2020)
  3. To characterize extracellular vesicles which transport HCC-derived CRNDE to TILs (3/30/2020, 3/15/2020)
  4. To delineate the molecular mechanism of CRNDE-CypB interaction in TILs (8/15/2020, 7/10/2020)

  Second year (9/16/2020 to 9/15/2021):

  5. To assess CRNDE effects on hepatocarcinogenesis (12/30/2020, 1/5/2021)
  6. To determine the downstream molecular targets of CRNDE-CypB complex (1/31/2021, 4/5/2021)
  7. To assess therapeutic efficacy of CRNDE specific ASOs, alone or in combination with checkpoint antibodies nivolumab/ipilimumab. (7/31/2021, 10% completion)

- Accomplishments under major goals

  1. To assess CRNDE effects on hepatocarcinogenesis.

     For this purpose, we modified the HCC hydrodynamic protocol by inclusion of CRNDE expression or shRNA plasmids into the Yap^{S127A}/β-$catenin^{\Delta N90}$/SB plasmid mixture solution for HDTV injection. Eight weeks (for CRNDE over-expression and Vector control groups) or twelve weeks (for shCRNDE and shControl groups) after hydrodynamic tail vein injection, mouse livers were recovered and the liver/body weight ratio for each group was measured. The results demonstrated that the average liver/body weight ratio of shCRNDE group is decreased by 50% when compared with that of shControl group (Fig6); and the average liver/body weight ratio of CRNDE over-expression group is increased by 40% compared with that of vector control group. These results suggest that CRNDE could significantly enhance hepatocarcinoma growth through inhibiting anti-tumor activities of TILs.

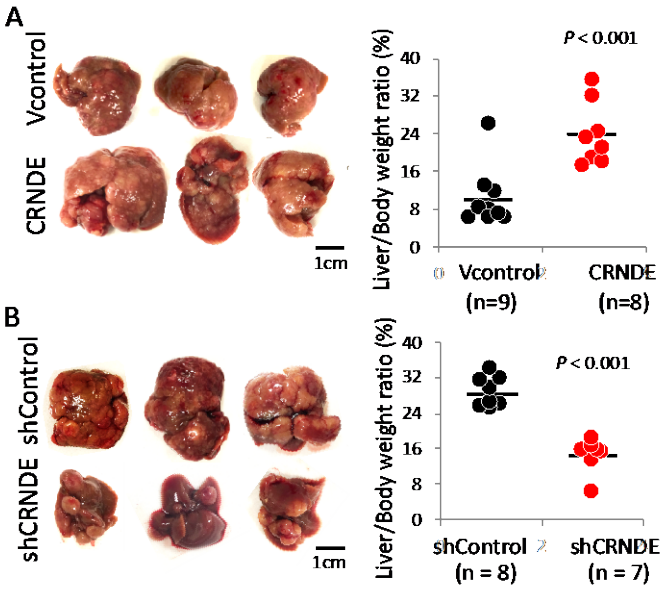

Fig6. ***CRNDE enhances hepatocarcinogenesis in mouse.*** *Mice were hydrodynamically injected with Yap^{S127A}/β-catenin$^{\Delta N90}$/SB plasmids in combination with CRNDE-expressing (**A**), shCRNDE (**B**) or their respective control plasmid. Mouse livers were recovered 8 weeks (for CRNDE over-expression) or 12 weeks (for CRNDE knockdown) after injection.*

2. To determine the effect of CRNDE on TIL functions by scRNA-seq.

   First, we induce the HCCs in C57BL/6 mice through hydrodynamic tail vein injection of Yap^{S127A}/β-catenin$^{\Delta N90}$/SB in combination with shCRNDE or shControl plasmids. After tumor fully developed (6-8 weeks later), mouse livers were perfused/digested with PBS/Collagenase D and harvested. Live non-parenchymal cells (NPCs, including TILs) were then isolated with method of mechanical homogenization and Iodixanol (20% in PBS) density gradient centrifugation. Purified NPCs were subjected to scRNA-seq.

   The results show that CRNDE depletion in HCC reduces TAM population and promotes CD8+T cell activation. The UMAP clusters were shown in Fig 2A. Results revealed that CRNDE depletion in HCC cells led to great reductions of all three clusters of monocyte-derived macrophages (MDMs) (12.2%, 8.6%, and 4.2% of total MDMs/Kupffer in control-HCC vs. 1.5%, 5.2%, and 1.3% of total MDMs/Kupffer in shCRNDE-HCC for MDM-1, -2, and -3 respectively, as circled in Fig 2A). Analyzing of single cell transcriptomes revealed that the infiltrating Kupffer cells and MDMs had gene expression signatures of M2-polarization (Fig 2B), which is one of the main features of tumor associated macrophage (TAM). Further analyses showed that MDM-1, the most reduced cell cluster in CRNDE-depleted HCC has the highest expression levels of typical TAM marker genes Trem and Stab1 (Fig 2C). Notably, targeting Trem2high/Stab1high TAMs with specific antibodies can greatly enhance therapeutic efficacy of check-point blockades (e.g., anti-PD1) in cancer treatment. Next, we checked the co-stimulatory and co-inhibitory checkpoint gene expression levels in CD8+T cells. As shown in Fig 2D, the levels of NFAT target-genes, Icos, Cd28 (including co-stimulator); Ctla4, Lag3 (co-inhibitor), Il4, Il2rb, and Nfatc1 were significantly increased in CD8+T-cells from CRNDE-depleted HCC as compared to corresponding cells in control-HCC. These results reiterate the involvements of CRNDE in shaping the tumor-facilitating, immunosuppressive TMEs leading to the remodeling of macrophage landscape and the inhibition of CD8+T-cell activity/cytotoxicity.

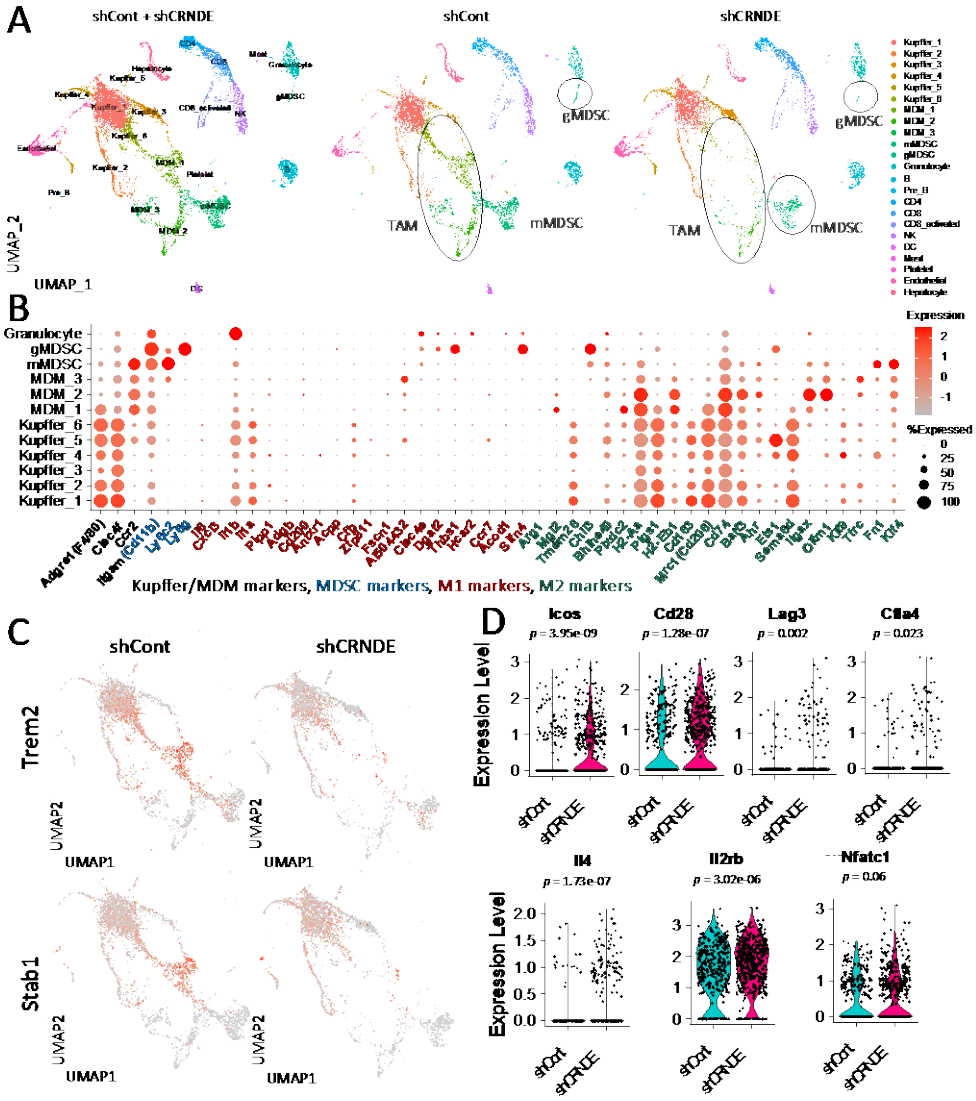

Fig 2. ***CRNDE effects on TAM and infiltrating CD8⁺ T-cell.*** *NPCs isolated from mouse HCCs (induced by Yap^{S127A}/β-$catenin^{\Delta N90}$ in combination with shCRNDE or shControl), were subjected to scRNA-seq. (**A**) UMAP plot demonstrating clusters identified in mouse NPCs. TAMs and MDSCs were denoted by circles. (**B**) Dot plot showed the expression of M1/M2 polarization marker genes in different clusters of MDMs/Kupffer cells. (**C**) TAM marker genes Trem2 and Stab1 levels in various clusters of MDMs/Kupffer cells. (**D**) Violin plots showed the NFAT target-gene levels in CD8+T cells from CRNDE-depleted HCC or control HCC.*

- Opportunities for training and professional development

  Nothing to report.

- How were the results disseminated to communities of interest?

  Nothing to report.

- Plan for next reporting period

  According the provide SOW, our plan for next year are the following:

  1. To complete the major task 6 "*To determine the downstream molecular targets of CRNDE-CypB complex*", we will measure the level and phosphorylation status of NFATs in Jurkat or TIL cell when the CRNDE level is manipulated. Then, we will explore the effect of CRNDE alteration on the protein-protein interaction between NFAT and Calcineurin. Finally, the possible calcium ion level change in Jurkat or TIL cells incurred by CRNDE level alteration will also be assessed.

2. To complete the major task 6 "*To assess therapeutic efficacy of CRNDE specific ASOs*", we will test the therapeutic efficacy of our selected, CRNDE-specific ASOs using HDTV or allograft mouse HCC model.

4. Impact

Nothing to report

5. Changes/Problems

Nothing to report

6. Products

- Journal publications

  Jinqiang Zhang, Weina Chen, Wenbo Ma, Kyoungsub Song, Sean Lee, Chang Han and Tong Wu. Epigenetic Silencing of 15-Hydroxyprostaglandin Dehydrogenase by Histone Methyltransferase EHMT2/G9a in Cholangiocarcinoma. Molecular Cancer Research. (in revision, predicted publication date: Nov 2021). Acknowledgement of federal support: Yes.

- Conference abstracts

1. Jinqiang Zhang, Weina Chen, Chang Han, Kyoungsub Song, Wenbo Ma, Tong Wu. Tumor-derived LncRNA CRNDE inhibits the cytotoxicity of tumor infiltering lymphocyte in Hepatocellular Carcinoma. AASLD Liver Meeting 2021 (Nov 12, 2021 - Nov 15, 2021). Acknowledgement of federal support: Yes.
2. Jinqiang Zhang, Weina Chen, Chang Han, Kyoungsub Song, Wenbo Ma, Tong Wu. The tumor-driving function of the histone methyl transferase EZH2 is augmented by HMGB1 in cholangiocarcinoma. AASLD Liver Meeting 2021 (Nov 12, 2021 - Nov 15, 2021). Acknowledgement of federal support: Yes.
3. Jinqiang Zhang, Weina Chen, Chang Han, Kyoungsub Song, Wenbo Ma, Tong Wu. Epigenetic silencing of 15-hydroxyprostaglandin dehydrogenase by histone methyltransferase ehmt2/g9a in cholangiocarcinoma. AASLD Liver Meeting 2021 (Nov 12, 2021 - Nov 15, 2021). Acknowledgement of federal support: Yes.

7. Participants & Other Collaborating Organizations

- Individuals have worked on the project

Name	Project role	Researcher identifier	Nearest person month worked	Contribution	Funding support
Jinqiang Zhang	No change				
Tong Wu	No change				
Weina Chen	No change				
Wenbo Ma	No change				

- Changes in the active other support of the PD/PI(s) or senior/key personnel since the last reporting

  Nothing to report

- Other organization

  Nothing to report

8. Special Reporting Requirements

Nothing to report

9. Appendices

None.