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AUTHOR'S VIEW

Epigenetic dysregulation by aberrant metabolism in renal cell carcinoma can be reversed with Ascorbic acid

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ABSTRACT

Our recently published study uncovered mechanisms and prognostic impact of aberrant DNA methylation/hydroxymethylation in clear cell renal cell carcinoma, and comprehensively explored the potential of Ascorbic acid in reversing the epigenetic aberrancy. This article provides a summary of the findings and their translational significance, and important considerations while testing Ascorbic acid as an anti-cancer agent.

Abbreviations- ccRCC: clear cell renal cell carcinoma; **TET**: Ten-Eleven Translocation; **5mC**: 5-methylcytosine; **5hmC**: 5-hydroxymethylcytosine; **L2HG**: l-2-hydroxyglutarate; **L2HGDH**: l-2-hydroxyglutarate dehydrogenase; **2-OG**: 2-Oxoglutarate; **AA**: Ascorbic acid

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Clear cell renal cell carcinoma (ccRCC) is an epigenetically unique solid tumor characterized by widespread DNA hypermethylation which plays a central role in its progression .[1–3] Although some cases of ccRCC had been previously shown to have a loss of 5-hydroxymethylcytosine (5hmC), its prognostic impact, mechanisms governing gain of 5-methylcytosine (5mC) and loss of 5hmC, and therapeutic targeting of this epigenetic aberrancy had not been fully explored.

Loss of 5hmc is an independent adverse prognostic biomarker in ccRCC

In our recent study,[4] through analysis of 576 primary ccRCC cases, we reported that loss of 5hmC is associated with aggressive clinicopathologic features and is an independent adverse prognostic factor in ccRCC. It also predicts a shortened time to metastatic disease after surgical resection for localized disease. We also showed that a grading of 5hmC immunohistochemistry (IHC) based on intensity (absent, mild, moderate and marked) or based on percent positive tumor cells, can be used as a strong tool to predict outcomes and could potentially be integrated in prognostic models, therapeutic decisions as well as clinical trial designs in the future. Given that this is a simple IHC test, it could potentially be adopted universally as a prognostic biomarker in ccRCC. Efforts are ongoing to determine if 5hmC can be a useful prognostic biomarker in other malignancies.

Loss of 5hmc in ccRCC is due to metabolic inhibition of TET enzymes

Ten-Eleven Translocation (TET) enzymes are dioxygenase enzymes involved in active demethylation through a series of oxidation steps, the first of which is 5mC to 5hmC. We showed that gain of 5mC and loss of 5hmC in ccRCC is not due to mutational or transcriptional inactivation of TET enzymes, but by their functional inactivation by l-2-hydroxyglutarate (L2HG), an oncometabolite that accumulates largely due to the deletion and under-expression of l-2-hydroxyglutarate dehydrogenase (*L2HGDH*). L2HG competes with 2-oxoglutarate (2OG), a necessary co-substrate of the TET enzymes. *L2HGDH* is located on chromosome 14q (along with Hypoxia-Inducible Factor-1α) and is deleted in 41% of all ccRCC. We found a strong correlation between loss of L2HGDH and loss of 5hmC in ccRCC (IHC). Furthermore, loss of *L2HGDH* conferred worse prognosis (The Cancer Genome Atlas) (*Figure 1. Schematic representation).*

Ascorbic acid restores 5hmc in ccRCC and inhibits ccrcc proliferation in vitro and in vivo

Ascorbic acid (AA) is a cofactor for the TET enzymes, reducing the enzyme-bound iron from Fe3+ to Fe2+. AA had been previously shown to cause TET-mediated demethylation of embryonal stem cells .[5] We, therefore, hypothesized that we could use AA as an epigenetic targeting agent in clear cell RCC given the genome-wide aberrant methylation. Indeed, we found that AA treatment increases TET activity, reduces 5mC and increases 5hmC in ccRCC. AA treatment was found to result in ccRCC proliferation inhibition in vitro and in a xenograft model in vivo. Histologic examination of the xenografts treated with intravenous AA revealed increased intra-tumoral 5hmC and enhanced differentiation.

l-2-hydroxyglutarate (L2HG) is an oncometabolite which accumulates in clear cell renal cell carcinoma (ccRCC)

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Auto-commentary on "Ascorbic acid-induced TET activation mitigates adverse hydroxymethylcytosine loss in renal cell carcinoma"

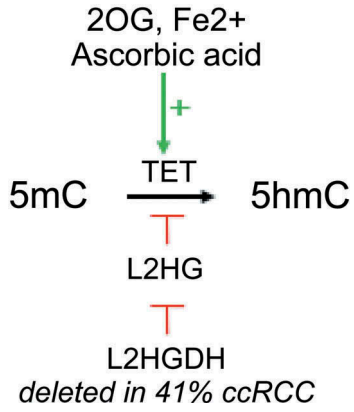

Figure 1. Schematic representation.

primarily due to the deletion and under-expression of l-2-hydroxyglutarate dehydrogenase (*L2HGDH*). L2HG inhibits the Ten-Eleven Translocation (TET) enzymes by competing with the natural co-substrate 2-oxoglutarate (2OG), thereby resulting in a gain of DNA 5-methylcytosine (5mC) and a loss of 5-hydroxymethylcytosine (5hmC). Loss of 5hmC is an independent adverse prognostic factor in ccRCC. Ascorbic acid activates the TET enzymes despite the accumulation of L2HG and increases 5hmC levels, thereby reversing the epigenetic aberrancy.

There were some important considerations and findings while studying the effects of Ascorbic acid as an anti-cancer agent in vitro and in vivo:

A. Is the in vitro effect from AA or H_2O_2? Although AA is an anti-oxidant, in the presence of free catalytic ions in culture media, it produces pro-oxidant hydrogen peroxide through the following reactions:

(1) Ascorbate reduces catalytic metal ions such as ferric ions to ferrous ions.

$$\mathbf{AscH^-}\ (\text{ascorbate}) + Fe^{3+} \rightarrow Asc^{\star -}\ (\text{ascorbate radical}) + Fe^{2+}$$

(2) The ferrous ions react with oxygen to form superoxide radical.

$$Fe^{2+} + O_2 \rightarrow Fe^{3+} + O_2^{\star -}$$

(3) Superoxide radicals then dismutes to H_2O_2 and O_2.

$$O_2^{\star -} + O_2^{\star -} + 2H^+ \rightarrow \mathbf{H_2O_2} + O_2$$

H_2O_2 is toxic to cancer cells. It is therefore important to neutralize H_2O_2 with catalase prior to studying epigenetic (or other cofactor related) effects of AA. This is particularly true with high doses (millimolar concentrations) of AA. Through catalase control and TET 1/2/3 knockdown experiments, we found that the demethylation effects of AA are independent of H_2O_2 and dependent on TET enzymes in ccRCC.

B. Interaction of AA with TET enzymes: It is very difficult to determine the concentration of AA needed for its maximal effect on the TET enzymes with conventional experiments. The reasons are several – Michaelis constant (Km) shifts of ascorbate transporters, decreased/mutated enzyme/transporter copy numbers, the rate of intracellular/extracellular oxidation, Km shifts and copy numbers of TET enzymes, etc.

We, therefore, used the fluorescence quenching technique with recombinant TET-2 and AA, which depends on conformational changes of the protein induced by its binding with AA. We found that >90% quenching of recombinant TET-2 fluorescence is obtained with 132μM AA. We also studied the dynamics of fluorescence quenching of the recombinant TET-2 with oncometabolite L2HG and co-substrate 2OG in the presence and absence of AA. We found that the quenching efficiency of 2OG is higher than that of L2HG, indicating that the substrate specificity of the TET-2 protein is 2OG over L2HG. Furthermore, the fluorescence quenching of the TET enzyme with AA was largely unaffected by the presence of oncometabolite L2HG, which suggested that in the presence of AA, the TET enzyme may be unaffected by L2HG. Indeed, ccRCC cell line 786-O had markedly higher intracellular L2HG levels compared to the immortalized kidney cell line HKC-8, and AA treatment of 786-O caused TET-dependent demethylation and increase in 5hmC.

C. *Dosing considerations*: It is important to consider the history and evolution of Ascorbic acid as an anti-cancer agent, and the available pre-clinical and clinical data while designing both in vitro and in vivo experiments with AA.

Oral AA has been tested as an anti-cancer agent and has failed.[6,7] There was no signal (either in the series by Cameron/Pauling in which oral AA was used after the first 7–10 days of intravenous AA treatment or in the Mayo studies in which only oral AA was used) to suggest that hypermethylated malignancies such as kidney cancer fared any better with oral AA treatment. On the other hand, pharmacologic AA is emerging as a promising agent in the treatment of established cancers, both in preclinical animal models (AA administered intraperitoneally) and more recently in early phase clinical trial data (AA administered intravenously at doses around 1g/kg 2–3 times/week) .[8]

Intraperitoneal and intravenous administration of ascorbate in mice has been shown to achieve 'pharmacologic' plasma concentrations in the millimolar range – over 100 times that with oral dosing – by bypassing the tight gastrointestinal regulation.[9,10] Intraperitoneally administered AA and not oral AA resulted in tumor shrinkage in a murine hepatoma model .[9]

It is therefore important to design in vitro and in vivo experiments taking into account the pharmacokinetics of parenteral AA in humans and mice (a cycle of 'bench to bedside and bedside to bench'). At these high concentrations of AA in vitro, it is essential to neutralize H_2O_2 with catalase to study non-free radical effects.

Our xenograft study was the first to use tail vein injections (5d/wk) to administer AA in order to further mimic intravenous AA treatment in humans.[4] The prior animal model studies with parenteral AA were done with intraperitoneal administration. Although the intraperitoneal route attains plasma concentrations much higher than that with oral administration, it is not as high as that with the IV route

with the same dose. The bioavailability fraction of intraperitoneal administration is around 0.62.[9]

D. *Epigenetic reprogramming or Oxidative stress*?: Two mechanisms of AA-induced anti-cancer activity have gained prominence: TET-mediated demethylation and H_2O_2-induced oxidative stress .[8] In vitro cancer cell cytotoxicity with short-term exposure to high dose AA is almost solely due to H_2O_2, given the complete reversal with catalase. However, it is important to recognize that for anti-oxidant AA to form pro-oxidant H_2O_2, free catalytic metal ions are required (as shown in reactions 1–3 above). While these are plentiful in vitro, they may be restricted in the tumor microenvironment. H_2O_2 has been shown to be generated in the extracellular fluid compartment but not within tumors in animal models .[10] In our xenograft study, [4] we showed that high dose intravenous AA increases 5hmC within ccRCC tumors as well as enhances differentiation. The increased intra-tumoral 5hmC, enhanced differentiation and delayed tumor growth with high-dose AA, when taken together, suggest that epigenetic reprogramming is an important mechanism of intravenous AA-induced anti-cancer activity.

Mice have a functional L-gulonolactone oxidase (GULO) enzyme which enables them to produce L-AA from L-Gulono-1,4-lactone. In humans, the GULO enzyme turned non-functional over the course of evolution, making AA an essential dietary vitamin. The finding of enhanced intra-tumoral 5hmC with intravenous AA despite the presence of a functional GULO enzyme suggests that supra-physiologic plasma concentrations of ascorbic acid are needed even for optimal cofactor functions of AA within tumors, not just for potential oxidative stress.

Statement of Translational significance

The above findings not only enhance our understanding of the epigenetic dysregulation in kidney cancer in terms of mechanisms and prognostic impact, but also provide the rationale for testing high dose intravenous Ascorbic acid in the clinical setting in this malignancy.

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