Restoration of 5-hydroxymethylcytosine by ascorbate blocks kidney tumour growth

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Abstract

Loss of 5-hydroxymethylcytosine (5hmC) occurs frequently in a wide variety of tumours, including clear-cell renal cell carcinoma (ccRCC). It remains unknown, however, whether the restoration of 5hmC patterns in tumours could have therapeutic efficacy. Here, we used sodium L-ascorbate (vitamin C, AsANa) and the oxidation-resistant form L-ascorbic acid 2-phosphate sesquimagnesium (APM) for the restoration of 5hmC patterns in ccRCC cells. At physiological concentrations, both show anti-tumour efficacy during long-term treatment in vitro and in vivo. Strikingly, global 5hmC patterns in ccRCC cells after treatment resemble those of normal kidney tissue, which is observed also in treated xenograft tumours, and in primary cells from a ccRCC patient. Further, RNA-seq data show that long-term treatment with vitamin C changes the transcriptome of ccRCC cells. Finally, APM treatment induces less non-specific cell damage and shows increased stability in mouse plasma compared to AsANa. Taken together, our study provides proof of concept for an epigenetic differentiation therapy of ccRCC with vitamin C, especially APM, at low doses by 5hmC reprogramming.

Keywords 5-hydroxymethylcytosine; clear-cell renal cell carcinoma; differentiation; epigenetic reprogramming; vitamin C
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Introduction

The lack of cellular differentiation is an important hallmark of many cancers [1]. Thus, a strategy which induces malignant cell differentiation may represent an attractive cancer therapy. Treatment of acute promyelocytic leukaemia (APL) with all-trans retinoic acid has been recognized as the best proof of principle for differentiation therapy [2]. Unfortunately, differentiation therapy for solid tumours remains limited. Thus, elucidating the mechanisms and developing effective differentiation-inducing agents are urgently needed.

Recently, it was found that 5-methylcytosine (5mC) can be oxidized to 5hmC by Ten-eleven translocation (TET) proteins, which belong to the family of Fe(II)/α-ketoglutarate-dependent dioxygenases [3]. Notably, recent evidence suggests that 5hmC levels correlate with the differentiation state of cells in hierarchically organized tissues, and that highly differentiated cells have the highest 5hmC levels, while less differentiated stem/progenitor cells have very low 5hmC levels [4]. These results suggest that 5hmC may regulate cell differentiation. Remarkably, 5hmC levels are dramatically reduced in a variety of human cancers, including ccRCC [5,6]. Consistently, our previous study showed that overexpressing IDH1 and pharmacologically elevating intracellular α-ketoglutarate could restore global 5hmC levels and suppress tumour growth in a xenograft model [5]. However, it remains to be determined whether the growth of ccRCC tumour cells can be inhibited by re-establishing 5hmC patterns versus a more differentiated state. Thus, we speculate that restoring 5hmC levels of tumour cells may result in a differentiation process similar to differentiation therapy in APL.

Recent studies have demonstrated that vitamin C regulates somatic cell reprogramming by promoting the catalytic activity of TET enzymes [7]. Vitamin C also modulates TET1 function and maintains a blastocyst-like state in ES cells [8,9]. Therefore, vitamin C may have an anti-tumour effect as an epigenetic reagent. Here, we explore whether vitamin C can induce 5hmC reprogramming and thereby a differentiated state in kidney tumour cells at physiological concentrations. A drawback of in vitro studies is that they do not take into account the tumour microenvironment, such as the presence of oxygen and iron, which can interfere with the potential therapeutic efficacy of vitamin C.
in vivo. Additionally, the production of reactive oxygen species (ROS) via oxidation of vitamin C appears to be a major underlying event, leading to the selective killing of cancer cells [10]. Thus, we also used APM, which is an oxidation-resistant vitamin C derivative [11], to evaluate whether the effect of vitamin C is dependent on ROS. Our data demonstrate that APM shows a comparable capacity to induce 5hmC reprogramming of kidney tumour cells vs. a normal kidney state as AsANa, but induces significantly less non-specific cell damage. Further, APM shows better stability in mouse plasma compared to AsANa. Thus, our study provides a proof of concept for an epigenetic differentiation therapy inducing 5hmC reprogramming in ccRCC by vitamin C and its derivatives.

Results

APM significantly increases 5hmC levels at physiological concentrations but with decreased cell damage compared to AsANa

We found that AsANa treatment for 24 h can increase 5mC levels in ccRCC cell lines at both physiological (100–250 μM) and pharmacological (0.5–10 mM) concentrations (Figs 1A and EV1A). However, because the physiological microenvironment, such as the presence of oxygen and iron, will also affect the activity of TET enzymes, we included an oxidation-resistant vitamin C derivative, APM. Notably, APM showed a similar capacity to that of AsANa in promoting 5mC in three ccRCC cell lines at the same concentration (Fig 1B). Because 5mC is an oxidized product of 5mC catalyzed by TET enzymes, we further examined the 5mC status after treatment with vitamin C. As expected, we observed a decrease in 5mC levels in both ccRCC cells and HK-2 cells (an immortalized proximal tubule epithelial cell line; Fig 1C). Strikingly, APM treatment for 24 h did not show significant cellular toxicity in ccRCC cell lines and HK-2 cells, even at a concentration of 10 mM (Fig 1D and E). However, AsANa at a concentration of more than 1 mM is toxic to both ccRCC cells and non-malignant HK-2 cells. Since production of ROS via oxidation of vitamin C appears to be a major underlying event, leading to the selective killing of cancer cells, APM as oxidation-resistant vitamin C derivative may generate less cellular toxicity, as it induces less ROS production. Consistent with this scenario, AsANa significantly increased H2O2 levels at mM concentrations in both ccRCC cell lines and APM treatment did not produce sufficient H2O2 even at 5 mM (Fig 1F). Notably, at physiological levels, both AsANa and APM produced no or minor H2O2, but induced significantly 5hmC restoration (Fig 1B and F). Therefore, we conclude that the epigenetic effects observed with both AsANa and APM at low doses are independent of H2O2.

Vitamin C inhibits the growth of ccRCC cells in a TET-dependent manner in vitro and in vivo

Next, we explored the functional consequences of the re-establishment of 5hmC levels in ccRCC cells. We found that both AsANa and APM had an inhibitory effect on both cell proliferation and migration in ccRCC cells (786-O and A498) and Caki-2 cells at physiological concentrations for 10 passages (Figs 2A and B, and EV1B). Then, we explored whether vitamin C altered the growth of 786-O and A498 cells in mice. Notably, vitamin C in the plasma of mice easily reached physiological levels (≈100 μM) upon intraperitoneal (IP) injection of low-dose vitamin C (0.5 g/kg for both AsANa and APM) once a day (Fig EV1C). As expected, APM showed better stability in mouse plasma compared to AsANa (Fig EV1C). Next, mice bearing established xenografts derived from 786-O and A498 cells were treated once a day with IP injection of low-dose vitamin C (0.5 g/kg) or PBS (control), for 6 and 4 weeks, respectively. We found that vitamin C treatment inhibited the growth of ccRCC xenografts (Fig 2C and D), and the 5mC levels were also significantly rescued both in AsANa- and in APM-treated xenograft tumours (Figs 2E and EV1D and E).

To further evaluate whether the potential therapeutic efficacy of vitamin C on ccRCC cells is dependent on TET activity, we first examined the relative levels of TET proteins in ccRCC cells. We found that the expression of TET2 was the highest among the TET genes in both 786-O and A498 cells (Fig 2F). We then generated two TET2 knockout cell clones using the CRISPR/Cas9 system (Fig EV1F and G). We found that knocking out TET2 in 786-O ccRCC cells can compromise the induction of 5mC upon vitamin C treatment (Fig 2G). Also the inhibition of cell proliferation upon vitamin C treatment was partially diminished in TET2 knockout cells (Fig 2H). However, TET2 knockout in 786-O cells did not completely block the establishment of intracellular 5mC by vitamin C treatment, suggesting that vitamin C can also restore 5mC catalyzed by other TET enzymes. Next, we used a pan-TET inhibitor, NiCl2 [12], to inhibit TET enzymes. NiCl2 treatment blocked vitamin C-induced 5hmC restoration in both 786-O and A498 cells (Fig 2I). As expected, the growth inhibition of ccRCC cells by
vitamin C was abolished by NiCl₂ treatment especially in 786-O cells (Fig 2J). However, we cannot rule out the possibility that NiCl₂ may have effects on other 2OG-dependent dioxygenases. Collectively, these results further showed that vitamin C treatment inhibited the growth of ccRCC cells at least partially by regulating TET activity.

Figure 1.
Figure 2.
Restoration of 5hmC patterns by vitamin C towards those of normal kidney cells in vitro

First, we explored the reprogrammed patterns of 5hmC upon vitamin C treatment. Samples without treatment (control), with AsNa or APM treatment for 10 passages (AsNa-P10, APM-P10) and after withdrawal from AsNa and APM for another 10 passages (AsNa-P20, APM-P20) were used to profile genome-wide 5hmC patterns by 5-hydroxymethylated DNA immunoprecipitation (hMeDIP)-seq (Fig 3A and Appendix Table S1). We found that global 5hmC levels in both 786-O and A498 cells can be stably maintained upon continuous treatment with vitamin C and are reversibly lost after withdrawal from vitamin C (Fig 3B).

Next, we examined whether 5hmC patterns restored by vitamin C treatment are found genome-wide or if this is a locus-specific effect. Unsupervised hierarchical clustering analysis showed that AsNa-P10 and APM-P10 grouped together, while AsNa-P20 and APM-P20 grouped with the mock control in both 786-O and A498 cells (Fig 3C). Additionally, 2,364 peaks were identified as vitamin C-restored 5hmC peaks (see Materials and Methods) in 786-O cells and 2,163 peaks in A498 cells (Fig EV2A and B). We observed a significant increase of 5hmC levels within the peaks compared to their up- and downstream regions in vitamin C-treated ccRCC cells in comparison with mock-treated cells (Figs 3D and EV2C). As exemplified by the SLC12A3 locus (significantly down-regulated in ccRCC [11]), the re-establishment of 5hmC patterns by vitamin C treatment is seen in both 786-O cells and A498 cells (Fig 3E). The hMeDIP-qPCR and methylated DNA immunoprecipitation (MeDIP)–qPCR results further confirmed the vitamin C-induced increase in 5hmC levels and concomitant decrease in 5mC levels (Fig 3F). These results suggested that the 5hmC patterns restored by vitamin C treatment are locus-specific in ccRCC cells.

Next, we evaluated the functional significance of the genes which consistently showed vitamin C-restored peaks in both 786-O and/or A498 ccRCC cells. Ingenuity Pathway Analysis (IPA) revealed multiple enriched pathways, such as molecular mechanisms of cancer, and signalling by Rho family GTPases and RhoGDI signalling (Figs 3G and EV2D). Strikingly, similar KEGG pathways were also identified for the genes that lost 5hmC during ccRCC tumorigenesis in our previously published data [5]. These results indicated that vitamin C treatment specifically promoted 5hmC patterns, which resemble those of normal kidney in ccRCC cells. Consistently, Pearson’s correlation analysis showed that vitamin C treatment restored 5hmC patterns in 786-O and A498 cells versus those of normal kidney tissues (Fig 3H). Collectively, vitamin C treatment reprogrammed ccRCC cells towards normal kidney cells.
Figure 3.

- **A**: Schematic showing the experimental setup.
- **B**: Heatmap showing 5hmC levels across different conditions.
- **C**: Hierarchical clustering of genes.
- **D**: Heatmaps showing gene expression changes.
- **E**: Heatmap showing expression changes across different conditions.
- **F**: Graphs showing %input changes for SLC12A3 in 786-O and A498.
- **G**: Bar chart showing top canonical pathways by IPA.
- **H**: Heatmap showing expression changes in kidney and tumor tissues.

These images and graphs illustrate the effects of vitamin C as an epigenetic agent in renal cancer cells (RCC).
Vitamin C re-establishes the 5hmC landscape in xenograft tumours and primary cells from a ccRCC patient

Next, we tested whether vitamin C can also restore 5hmC patterns in 786-O xenograft tumours and primary cells from ccRCC patients. First, we examined 5hmC patterns in four randomly selected vitamin C-treated xenograft tumours (two from AsANa and two from APM) and one mock control xenograft tumour by hMeDIP-seq (Appendix Table S1). Consistently, we found that vitamin C treatments can also restore the 5hmC patterns of xenograft tumours (Fig 4A). The genes with restored peaks in 786-O xenograft tumours were similar to those identified in cultured 786-O cells (Fig EV2D and Dataset EV1).

Additionally, we also treated primary tumour cells and normal kidney cells from a ccRCC patient with vitamin C and examined global 5hmC level and pattern with dot blot and hMeDIP-seq, respectively. Notably, both AsANa and APM can specifically restore the 5hmC pattern of primary cells from a ccRCC patient to that of normal kidney cells (Fig 4B and C). IPA of the 198 genes that were consistently restored by vitamin C in cell lines, xenograft tumours and primary cells showed enrichment for tumour-related pathways (Figs 4D and EV2E, and Datasets EV1 and EV2). The heatmap shows that 1,016 5hmC peaks were lost in tumour tissue compared to normal kidney tissue and restored by vitamin C treatment to the level of normal kidney cells in primary tumour cell culture (Fig EV2F). One example is the ASPSCR1 locus [14], which is one of several reported partner genes fused with TFE3 in Xp11 (TFE3) by translocation in renal cell carcinomas (Fig 4E). The reciprocal changes of 5hmC and 5mC at this locus were further validated by hMeDIP–qPCR and MeDIP–qPCR (Fig 4F). Collectively, vitamin C treatment induces changes of 5hmC patterns towards those of normal kidney in xenograft tumours and primary cells from a ccRCC patient.

Vitamin C shifts the transcriptome of ccRCC cells

Next, we examined ccRCC phenotype changes at the global transcriptome level after treatment of vitamin C for 10 passages (Appendix Table S2). Eighty-one genes differentially expressed after prolonged treatment of vitamin C were identified by DESeq2. Of the 81 genes, 51 genes were up-regulated and the rest were down-regulated (Dataset EV3). Due to potential secondary effects in the long-term treatment, we only found 11 out of 81 genes in which the 5hmC pattern was also restored. Next, we performed gene set enrichment analysis (GSEA) to identify biological consequences of long-term vitamin C treatment. Strikingly, the most notable genes positively enriched in vitamin C-treated cells belong to multiple metabolic pathways, such as peroxisome and pentose phosphate pathways (Fig 5A). In contrast, the most notable gene sets negatively enriched in vitamin C-treated cells include DNA replication and mismatch repair genes (Fig 5B). To further examine whether vitamin C shifted the transcriptome of ccRCC cells, which may link to the clinical features, we examined the association between subtypes defined by mRNA-seq of ccRCC patients with clinical features from The Cancer Genome Atlas (http://gdcac.broadinstitute.org/). We found patients with subtype 2 had the best overall survival (Fig 5C). Consistently, the gene sets enriched in this group of patients were also linked to metabolic pathways (Fig 5D). Collectively, vitamin C shifts the gene expression patterns of multiple genes related to metabolic pathways which are associated with better overall survival in patients.

Vitamin C-restored 5hmC peaks occur preferentially at enhancers, especially super-enhancers

Distal regulatory elements, including enhancers and super-enhancers, play a critical role in defining tissue identity. Thus, we further explored whether vitamin C restored 5hmC patterns versus the normal kidney state through the re-establishment of distal regulatory elements. Typical enhancers were identified as H3K27ac-enriched regions, and super-enhancers were identified using the ROSE algorithm (Fig 6A; see Materials and Methods). We identified 14,519 potential typical enhancers and 677 potential super-enhancers with median lengths of 1,600 and 36,000 bp, respectively (Fig 6B). We consistently found that vitamin C-restored 5hmC peaks were enriched in both enhancer and super-enhancer regions, but not in intergenic regions (Fig 6C). Two representative super-enhancer- and typical enhancer-associated genomic loci, MYH9 and CELSR1, are shown in Fig 6D. Both genes are required for kidney development and diseases [15,16]. The reciprocal changes of 5hmC and 5mC in the locus were further validated by hMeDIP–qPCR and MeDIP–qPCR (Fig 6E).

Since super-enhancers are large clusters of transcriptional enhancers that drive the expression of genes that define tissue identity, we used the GREAT tool to predict functions of the super-enhancers which overlapped with vitamin C-restored 5hmC peaks (GREAT; http://bejerano.stanford.edu/great/public/html/index.php). A total of 284 genes were assigned as super-enhancer-associated genes. The most significantly enriched MSigDB pathways of these genes are as follows: HIF-1-alpha transcription
Figure 4.
factor network, signalling mediated by p38-alpha/p38-beta and IL-6-mediated signalling events (Fig 6F). Strikingly, all these pathways have been shown to be involved in embryonic vascular development, which is crucial both for kidney development and for renal cell carcinoma [17]. Collectively, these results suggested that vitamin C restored the normal kidney state at least partially through remodelling distal regulatory elements such as enhancers and super-enhancers.

Discussion

Cancer therapy with vitamin C has a controversial history. While some early studies indicated that vitamin C has anti-tumour activity, including a case report on ccRCC treatment [18–20], others have shown that it has little effect [21,22]. Recent studies suggested that the contradictory clinical data may be explained by differences in the administration route; millimolar vitamin C plasma concentrations cytotoxic to cancer cells are only achievable via intravenous administration and not via oral administration [23–26]. Recent studies also showed that vitamin C is quickly oxidized to dehydroascorbate (DHA) in cell culture (half-life of approximately 70 min), and production of ROS via oxidation of high-dose vitamin C appears to be a major underlying event, leading to the selective killing of cancer cells [27]. However, studies also showed that vitamin C as a co-factor can increase 5hmC and promote TET-dependent DNA demethylation [28,29]. Particularly, a recent study showed that vitamin C can block the serial re-plating capacity of haematopoietic stem and progenitor cells (HSPCs) when catalase, an enzyme that can decompose H2O2 to water and oxygen, was included with...
Figure 6.
vitamin C in the media [30]. Consistent with this, vitamin C can also cause epigenetic effects in lymphoma cell lines, which is independent of H2O2 [10,31]. Collectively, vitamin C may have dual effects on neoplastic cells, including differentiation induction upon low-dose prolonged exposure and cytotoxicity at high doses. Consistent with this scenario, a phase 1 study using prolonged exposure schedules at low doses of the hypomethylating agent 5-aza-2’-deoxycytidine (decitabine) in haematopoietic malignancies showed that decitabine is effective in myeloid malignancies, and that low doses are as effective or even more effective than higher doses [32].

In this study, we provide proof of concept for an epigenetic differentiation therapy using both AsA and APM at low-dose prolonged exposure inducing 5hmC reprogramming in ccRCC. We used an oxidation-resistant vitamin C derivative, APM, to show that the observed epigenetic effects are independent of H2O2. Strikingly, APM is highly resistant to degradation into AsA even at neutral pH, but is easily degraded into AsA in the presence of phosphatase from living tissues [33,34]. Consistent with this scenario, we found that APM showed better stability in mouse plasma compared to AsA. APM is broadly used in cosmetics. A double-masked, randomized, controlled clinical trial has shown that the regular application of an APM-containing dentifrice could reduce gingival inflammation [35]. Collectively, low-dose prolonged exposure to vitamin C, especially APM, may be effective in the treatment of kidney cancers, and low doses may be as effective as or more effective than high doses.

Even though it is unclear whether the results we have observed will translate to human kidney tumours, our findings on the differential mechanisms of vitamin C warrant future investigation through clinical trials. Limitations of this study include the relatively small number of ccRCC cell lines and patient samples used, owing to the limited availability of fresh tissues from patients, and limitations regarding the tissues’ quality and quantity for genome-wide sequencing and analyses. However, our observations serve as important hypothesis-generating findings that may initiate new clinical trials for kidney cancer, especially using oxidation-resistant vitamin C derivatives.

Materials and Methods

Cell culture and reagents

786-O and A498 cells were maintained in DMEM (high glucose) medium. 769-P cells were maintained in RPMI-1640 medium, and Caki-2 cells were maintained in McCoy’s 5a medium. HK-2 cells were maintained in low-glucose DMEM (high glucose) medium. 769-P cells were maintained in RPMI-1640 medium, and 786-O and A498 cells were maintained in DMEM (high glucose) medium. Caki-2 cells were maintained in McCoy’s 5a medium. HK-2 cells were cultured with sodium L-ascorbate (Sigma, #A4034) or L-ascorbic acid (Sigma, #A9960) as indicated. Catalase was obtained from Sigma (C1345). The nickel(II) chloride hexahydrate was obtained from J&K (Catalogue No. 486536). Antibodies used for immunohistochemistry, dot blot and Western blot were as follows: anti-5mC antibody (Active Motif, 39769), anti-5mC antibody (ZYMO RESEARCH, #A3001-200), anti-TET2 rabbit polyclonal antibody (Abcam, ab94580) and anti-actin monoclonal antibody (Proteintech, #6609-1-ig). The ΔΔCt method was used to analyse TET mRNA levels relative to TET1. The primer sequences are listed in Appendix Table S3. The C, 5mC and 5hmC standards were obtained from Zymo Research (D5405).

MTS cell viability assay, apoptosis assay, H2O2 measurement, cell proliferation assay and wound-healing assays

Cell viability assay was assessed using the CellTiter 96® AQ One Solution Reagent (Promega) according to the manufacturer’s instructions. Apoptosis assay was performed by FACS with Annexin V staining. H2O2 concentrations were measured using Amplex® Red Hydrogen Peroxide/Peroxidase Assay Kit (#A22188) following the manufacturer’s instructions. Cell proliferation assay was performed on the IncuCyte® ZOOM Live-Cell Analysis System (Essen BioScience). Cell proliferation was measured by analysing the occupied area (% confluence) of cell images over time. To measure the ability of cell migration, we performed wound-healing assays in ccRCC cell lines. The cell wound was created using an Essen ZOOM Live-Cell Analysis System (Essen BioScience). Cell proliferation was measured by analysing the occupied area (% confluence) of cell images over time. To measure the ability of cell migration, we performed wound-healing assays in ccRCC cell lines. The cell wound was created using an Essen 96-well WoundMaker, and the wound closure (%) was calculated as follows: Wound Closure (%) = (Wound_Area_0 h – Wound_Area_final)/Wound_Area_0 h × 100%.

Generation of the TET2 knockout cell line

The TET2 knockout 786-O cell line was established using the CRISPR/Cas9 system. The sgTET2 sequence was obtained from a previous study [36]. Oligonucleotides encoding the sgRNA were cloned into a BsmBI-digested pLenti-CRISPRv2 vector, and lentiviruses were generated. Lentivirus-infected 786-O cells were selected and TET2 knockout efficiency was assessed by Western blot. 786-O knockout pool was seeded in 10-cm dish, and single clones were picked. The knockout efficiency of TET2 in each clone was measured by Western blot.
Measurement of vitamin C in plasma of nude mice by LC/MS

The procedure of vitamin C measurement in plasma has been described in a recent study [27]. Briefly, plasma samples were incubated with 2.5 mM MBB in CH₃OH:H₂O (80:20) at room temperature for 30 min, then diluted with CH₃CN:H₂O (70:30) and centrifuged to pellet precipitated proteins. The supernatants were transferred to autosampler vials with 5 μl solution injection for analysis by LC/MS. Determination of AsANa and APM was carried out using an HPLC system equipped with an Acclaim 120 C18 column of 2.1 × 150 mm and 3 μm particle size, 120Å. The column was maintained at 20°C. The mobile phase was composed of methanol and 50 mM ammonium formate in gradient elution and dosed at the flow rate of 0.2 ml/min. The mass spectral analysis was performed on a 6,460 triple quadrupole mass spectrometer from Agilent equipped with an ESI interface. AsANa and APM were detected in negative mode ([M-H⁻]) with m/z = 175 and m/z = 254.9, respectively. Ion acquisition was accomplished in the MRM mode.

Hierarchical cluster and correlation analysis

Non-duplicate reads from the input and each hMeDIP sample were counted in each 10-kb bin and normalized to reads per 10 million reads (RP10M). The enrichment value of each bin was calculated using the following formula: RP10M_{hMeDIP}/RP10M_{input}; and then log2-transformed. The TAB-seq data of the two patients are from our previous study. 5hmC levels of every CpG site in each bin were summed and divided by the total CpG numbers in that bin as the 5hmC ratio in the tissues. Unsupervised hierarchical clustering was performed across all samples in 786-O and A498 cells based on Euclidean distance and complete linkage after z-score standardization of the log2 (enrichment value) for each bin. The correlation coefficient was calculated across all samples, including the two patients’ data, xenograft data and also a new ccRCC patient’s data, to broadly assess patterns of 5hmC distribution genome-wide using Pearson’s correlation, which were visualized as a heatmap.

RNA-seq and gene set enrichment analysis (GSEA)

The KAPA Stranded RNA-Seq Library Preparation Kit was used to construct RNA-seq libraries according to the manufacturer’s instructions. Sequencing reads were aligned to the human genome (hg19) each sample were determined by the MACS program (v.2.1.0, default settings) [37]. To identify the vitamin C-induced 5hmC regions in 786-O and A498 cells, the peaks were called in the following four paired samples: AsANa-P10 vs. control, AsANa-P10 vs. AsANa-P20, APM-P10 vs. control, and APM-P10 vs. APM-P20. P10 represented cells treated with AsANa or APM for 10 passages. And P20 represented withdrawal of AsANa or APM from P10 cells for another 10 passages. The candidate peaks were filtered with FDR < 0.05, and the intersected regions among these four pairs in 786-O cells were then annotated using the CEAS program against the hg19 human genome. However, the baseline level of 5hmC in A498 was higher than in 786-O (Fig EV2B), and we applied a less stringent cut-off to call the restored peaks in A498. The peaks were called in the following two paired samples: AsANa-P10 vs. control and/or AsANa-P20; and APM-P10 vs. control and/or APM-P20. The intersected peaks of these two pairs were called as vitamin C-restored peaks in A498. The peak-associated genes were assigned if the peak localized within 3 kb of the gene.

Hierarchical cluster and correlation analysis

Non-duplicate reads from the input and each hMeDIP sample were counted in each 10-kb bin and normalized to reads per 10 million reads (RP10M). The enrichment value of each bin was calculated using the following formula: RP10M_{hMeDIP}/RP10M_{input}; and then log2-transformed. The TAB-seq data of the two patients are from our previous study. 5hmC levels of every CpG site in each bin were summed and divided by the total CpG numbers in that bin as the 5hmC ratio in the tissues. Unsupervised hierarchical clustering was performed across all samples in 786-O and A498 cells based on Euclidean distance and complete linkage after z-score standardization of the log2 (enrichment value) for each bin. The correlation coefficient was calculated across all samples, including the two patients’ data, xenograft data and also a new ccRCC patient’s data, to broadly assess patterns of 5hmC distribution genome-wide using Pearson’s correlation, which were visualized as a heatmap.

Definition of enhancers and super-enhancers

The super-enhancers in adult normal kidney tissue (Roadmap, H3K27ac ChIP-seq data) were identified using the ROSE algorithm described in a previously published study [38]. The enhancer was assigned to the nearest gene.

The enrichment score of the 5hmC peaks in different genomic regions

The known genomic features were downloaded from UCSC Tables for hg19 (Exon, Intron and RefSeq Intergenic region), and enrichment scores were calculated as follows: # observed peaks/# Expected peaks. The # Expected peaks are determined as follows: Total peaks*The size of the genomic region/The size of the genome, where # represents the number.
by using the TopHat program (v2.1.1) with the default parameters. Total read counts for each protein-coding gene were extracted using HTSeq (version 0.6.0) and then loaded into R package DESeq2 to calculate differentially expressed genes with cut-off of fold change ≥ 1.5 and FDR < 0.05. Gene set enrichment analysis (GSEA) was performed using C2 (curated gene sets) collections.

Statistical analysis

All statistical analysis was performed on at least three independent replicates. Unless otherwise mentioned, results are shown as mean ± s.d. and statistical significance was determined by a two-tailed Student’s t-test for two-group comparison. Significance in all figures is indicated as follows: N.S. P > 0.05; *P < 0.05; **P < 0.01; and ***P < 0.001.

Data availability

The raw sequence data reported in this paper have been deposited in the Genome Sequence Archive in Big Data Center [39], Beijing Institute of Genomics (BIG), Chinese Academy of Sciences, under accession number PRJCA000352.

Expanded View for this article is available online.

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Author contributions

WC, LZ and XL conceptualized the project. GG, DP and ZXu conceived the in vitro experiments and analysed the data. GG, DP and YZ conceived and performed most of the experiments in animals and analysed the data. QH conceived the ShmC staining and analysed the data. BG and ZXi collected the samples from patients. WC, GG and DP wrote the manuscript, with inputs from other co-authors.

Conflict of interest

The authors declare that they have no conflict of interest.

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