ACTA ABP BIOCHIMICA POLONICA

Vol. 70, No 1/2023
145–152
https://doi.org/10.18388/abp.2020_6367

Regular paper

Hexafluoro-2-propanol represses colorectal cancer proliferation by regulating transaminases

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Hexafluoro-2-propanol (HFIP) is a metabolite of sevoflurane used as part of the general anaesthesia technique. This study aims to investigate the effect of HFIP in colorectal cancer (CRC) and the regulation of associated genes. The differential expressed genes (DEGs) with HFIP treatment were analysed based on GEO dataset GSE56256. Due to the restricted number of studies performing RNA-Seq in CRC with HFIP treatment, we selected a CRC patient cohort (GSE23878) to obtain DEGs that were compared with DEGs from GSE56256. The DEGs with opposite expression patterns in these two GEO datasets indicate that the genes mediate the function of HFIP, thereby repress the progression of CRC. The DEGs from these two GEO datasets were analysed independently. We obtained 10 up-regulated genes in GSE23878 (CRC patient cohort) were also down-regulated in GSE56256 (HFIP cohort), therefore, these 10 genes may function as tumor suppressors in CRC and may be involved in the function of HFIP. STRING analysis demonstrated an interaction in GPT2, PSAT1 and SLC7A11. The high expression of GPT2, PSAT1 and SLC7A11 were confirmed in TCGA-COAD datasets and in CRC cells. HFIP treatment repressed GPT2, PSAT1 and SLC7A11, which resulted in an inhibited proliferation and colony formation ability of DLD-1 and HCT-116 cells. Silencing GPT2, PSAT1 and SLC7A11 showed similar effect compared to HFIP treatment. Overexpression of GPT2, PSAT1 and SLC7A11 abrogated the effect of HFIP. In conclusion, the sevoflurane metabolite HFIP plays a cancer suppression role depending on cell proliferation in colorectal cancer through the down-regulation of GPT2, PSAT1 and SLC7A11 expression.

Key words: hexafluoro-2-propanol, transaminase, colorectal cancer

Received: 18 May, 2022; revised: 16 October, 2022; accepted: 08 January, 2023; available on-line: 23 February, 2023

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Abbreviations: AKG, α-ketoglutarate; CRC, colorectal cancer; DEG, differentially expressed gene; HFIP, hexafluoro-2-propanol; LPS, lipopolysaccharide

INTRODUCTION

Sevoflurane is a commonly used anaesthetic because of the safety with fewer side effects compared with other inhalation anaesthetics (Kanaya *et al.*, 2014). Studies confirmed that volatile anaesthetics influence cancer cells and show potential regulatory effect on a variety of tumours (Sumi *et al.*, 2019; Zhao *et al.*, 2020). During the metabolism of sevoflurane, water-soluble hexafluoro-2-propanol (HFIP) presents as a primary metabolite of sevoflurane (Buratti *et al.*, 2002). Previous researches have shown that HFIP attenuates inflammation *in vitro* by repressing related inflammatory mediators (Urner *et al.*, 2015). However, few studies investigated the effect of HFIP on cancer progression especially on colorectal cancer, therefore, we demonstrated the function of HFIP in colorectal cancer cells and aimed to illustrate the potential molecular mechanisms.

The growth of mammalian cells is supported by nutrient sources including glucose and glutamine, which are involved in energy production and are related to cancer progression (Hosios *et al.*, 2016). Most cancer cells proliferate depending on glutamine. Glutamine starvation results in growth inhibition. GPT2 is a type of glutamic pyruvate transaminase catalysing the reversible transamination between alanine and α-ketoglutarate (AKG) generating pyruvate and glutamate (Welch, 1975). Previous studies have shown that GPT2, PSAT1 and SLC7A11 are involved in the progression of different cancers: GPT2 is highly expressed in cancers, such as breast cancer and pancreatic cancer (Cao *et al.*, 2017). Therefore, GPT2 is a potential biomarker and target in cancer therapy. PSAT1 was identified highly expressed in various cancers including colorectal cancer and non-small cell lung cancer (Qian *et al.*, 2017; Lodovichi *et al.*, 2019). Studies demonstrated that PSAT1 is involved in serine biosynthesis and mediates cell cycle progression, which implies a promising target for anticancer therapy. In the regulation of nutrient dependency of cancer cells, SLC7A11 plays emerging roles which maintain redox homeostasis (Koppula *et al.*, 2018). In colorectal cancer stem cells (CSCs), SLC7A11 has a higher level compared to colorectal cancer cells. Repression of SLC7A11 sensitizes colorectal CSCs to ferroptosis and chemotherapy drugs (Xu *et al.*, 2020). Furthermore, repression of SLC7A11 inhibits metastasis of pancreatic cancer by blocking the PI3K/Akt signaling pathway (Zhang *et al.*, 2021). The pro-death functions of SLC7A11 happen under glucose starvation (Karunakaran *et al.*, 2008). Overexpression of SLC7A11 confers resistance to MEK and BRAF inhibitors in *BRAF*^{V600E} mutant melanoma via increasing intracellular GSH contents (Wang *et al.*, 2018). Cancer cells employ up-regulated SLC7A11 to mediate cystine uptake, which results in better capabilities of cancer cells to survive in oxidative stress conditions (Koppula *et al.*, 2018). SCL7A11 provides a novel and effective therapeutic strategy for cancer treatment.

In our study, the regulation of GPT2, PSAT1 and SLC7A11 by HFIP was investigated in colorectal cancer. This study showed that HFIP repressed colorectal cancer proliferation *via* regulating glutamine metabolism related genes.

MATERIAL AND METHODS

Cell culture and treatment

Human colorectal cancer cell lines DLD-1, HCT-116 and RKO were obtained from ATCC. Human colon epithelium cell HCoEpiC was purchased from National Collection of Authenticated Cell Culture. Cells were cultivated in McCoy's 5A medium containing 10% fetal bovine serum (FBS), penicillin 100 units/ml and streptomycin 100 ug/ml with 5% CO_2 in 37°C. HFIP (105528) was purchased from Sigma Aldrich and dissolved in DMSO to a stock concentration of 100 mM. Working concentration of 50 mM was achieved by diluting HFIP stock solution with HBSS. Before different experiments cells were treated with HFIP for 12 h.

Transfection

GPT2, PSAT1 and SLC7A11 pcDNA3.1 overexpressing vectors were designed and constructed by BGI (Beijing, China). Specific siRNAs of GPT2, PSAT1 and SLC7A11 were purchased from QIAGEN (Germany). For transfection, cells were seeded into 6-well plate at the density of 5×10^5 cells/well and cultivated to at least 70% confluence. Vectors were transfected with Lipofectamine 2000, and siRNAs were transfected with Lipofectamine RNAiMAX (Thermo Fisher, USA) at 1:1 ratio (v/v) according to the protocol provided by the manufacturer. The final concentration of vector and siRNA were 2 μg/ml and 25 nM, respectively.

RNA isolation and quantitative real-time PCR (q-PCR)

Total RNAs were isolated and purified by High Pure RNA Isolation Kit (Roche, Switzerland) based on the instruction of the kit. 100 ng of isolated RNA was used for reverse transcription with verso cDNA synthesis kit (Thermo Fisher, USA). Q-PCR was performed with SYBR® Premix Ex Taq™ (RR420A; Takara) in Roche LightCycler 480 system. The data were normalized to GAPDH and calculated using $2^{-\Delta\Delta Ct}$ value. Primers used in q-PCR were listed in Table 1.

Table 1.. Primers used in q-PCR analysis

Genes	Sequence 5′-3′
GPT2 F	GTGATGGCACTATGCACCTAC
GPT2 R	TTCACGGATGCAGTTGACACC
PSAT1 F	TGCCGCACTCAGTGTTGTTAG
PSAT1 R	GCAATTCCCGCACAAGATTCT
SLC7A11 F	TCTCCAAAGGAGGTTACCTGC
SLC7A11 R	AGACTCCCCTCAGTAAAGTGAC
GAPDH F	CTGGGCTACACTGAGCACC
GAPDH R	AAGTGGTCGTTGAGGGCAATG

Western blot

Total protein was isolated from colorectal cancer cells after different treatments by applying RIPA lysis buffer. Protein concentrations of each group were quantified with BCA kit based on the instructions. Total 40 μg of protein was loaded onto 10% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE). Electrophoresis conditions were 120 V for 120 min. It was then transferred to a polyvinylidene difluoride membrane (300 mA, 90 min). Membranes were blocked by 5% milk in 1×TBST and then incubated with primary antibodies GPT2 (1:1000, Life, USA), PSAT1 (1:1000, Santacruz, USA), SLC7A11 (1:1000, Abcam, USA) and β-actin (1:2000, Abcam, USA) overnight at 4°C.

MTT assay

For cell proliferation rate, after different treatments, cells were seeded in 96-well plates in 100 μl of medium. Three parallel wells were assigned for each time points. MTT solution (5 mg/ml) was added to each well and incubation continued for 4 hours. It was followed by removing medium and washing with PBS twice. Afterwards, 150 μl DMSO was added into each well and then plate was shaken for 10 min. OD values were measured at 490 nm wavelength.

Colony formation assay

After different treatments, cells were seeded into 6-well pate at the density of 500 cells/well and cultivated to 3 weeks until cell colonies could be observed by eye. Cells were fixed with methanol for 15 min and then stained with crystal violet for 30 min. After staining, cells were washed under running water and taken pictures of by camera. Colony numbers were counted by using Image J software.

Bioinformatic analysis

GSE56256 and GSE23878 were obtained from GEO database (https://www.ncbi.nlm.nih.gov/geo/), and the download data format was MINIML. GSE56256 includes two groups of lipopolysaccharides (LPS)/HFIP treated endothelial cells and three groups of LPS treated endothelial cells (Urner *et al.*, 2015). The array profiling was performed on the platform of Agilent-028004 SurePrint G3 Human GE 8x60K Microarray. GSE23878 contains 35 colorectal cancer patient samples and 24 normal samples (Uddin *et al.*, 2011). The cDNA microarray was performed on the [HG-U133_Plus_2] Affymetrix Human Genome U133 Plus 2.0 Array. Limma package (version: 3.40.2) of R software was used to study the differential expression of mRNAs. The adjusted *P*-value was analysed to correct for false positive results in GEO datasets. "Adjusted $P<0.05$ and $\left|\text{Log}_2[\text{Fold Change}]\right| >1$" were defined as the thresholds for the screening of differential expression of mRNAs. To further confirm the underlying function of potential targets, the data were analyzed by functional enrichment. To better understand the carcinogenesis of mRNA, ClusterProfiler package (version: 3.18.0) in R was employed to analyze the GO function of potential targets and enrich the KEGG pathway. The box plot is implemented by the R software package ggplot2; PCA graphs are drawn by R software package ggplot2; the heat map is displayed by the R software package heatmap. All the above analysis methods and R package were implemented by R foundation for statistical computing (2020) version 4.0.3.

Statistics

SPSS 21.0 was used to calculate all the values (means ± standard error of the mean). Statistical analyses were analysed with Student's t test in two-groups comparison. One-way analysis of variance (ANOVA) test was used to verify the significance among experimental groups. The statistical significance was $P<0.05$. All experiments were performed in triplicate.

RESULTS

Analysis of differentially expressed genes with HFIP treatment

Since few studies performed next generation analysis of HFIP treatment in colorectal cancer cells or patients, here we selected a GEO dataset GSE56256 which investigated that HFIP as an anti-inflammation reagent in lipopolysaccharides (LPS) induced inflammatory response in lung cancer. Therefore, we could obtain genes that are regulated by HFIP. We compared the duplicate of LPS-HFIP co-treatment groups to the triplicate of LPS treatment groups. A total of 5583 genes were down-regulated with Log_2[Fold Change] <–1 while a total of 54 genes were up-regulated with f Log_2[Fold Change] >1 after HFIP treatment (Fig. 1A and 1B). From the results, DEGs were mainly enriched in down-regulated group. Next, DEGs were put into KEGG signalling pathway

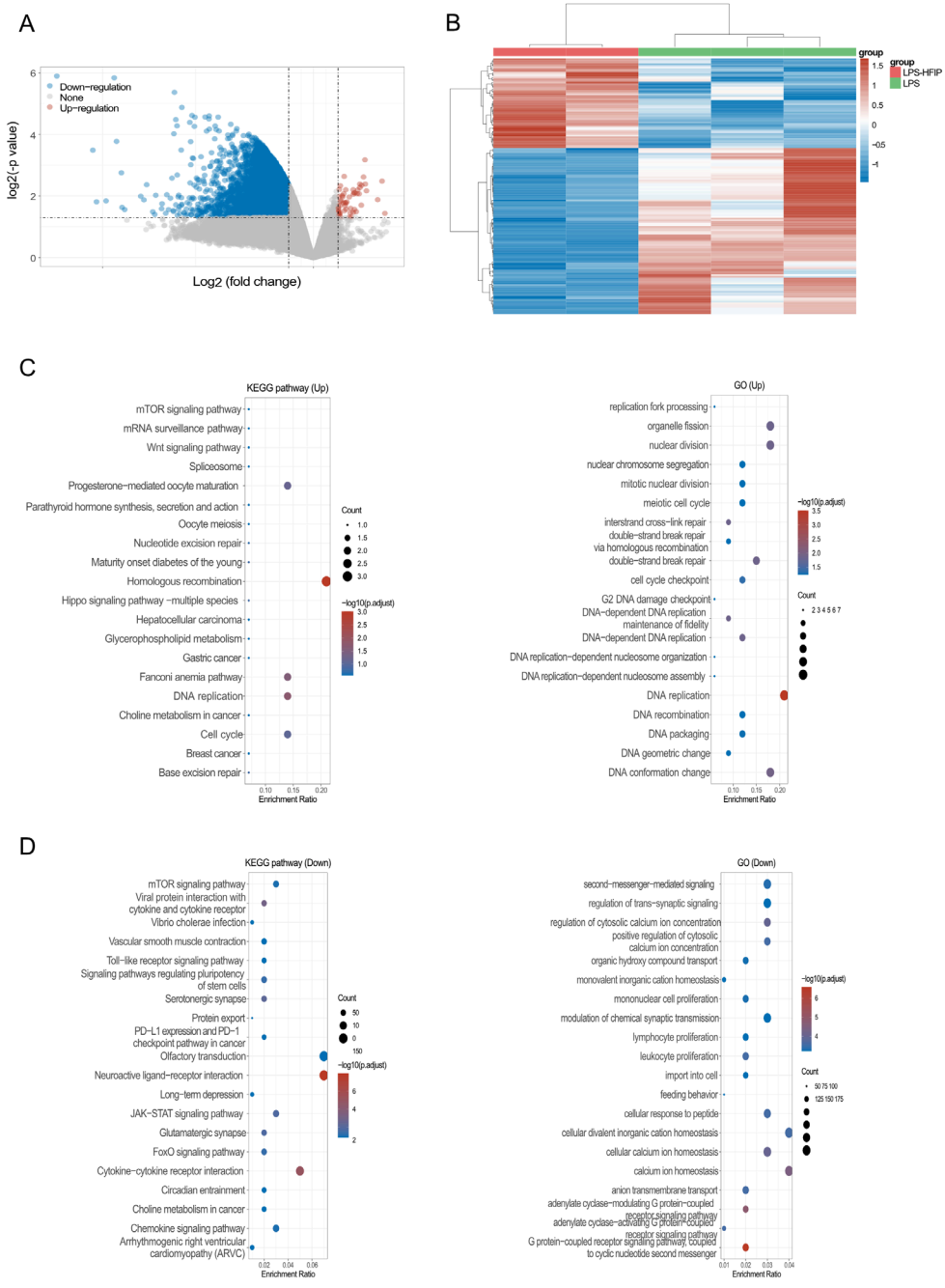

Figure 1. Analysis of differential expressed genes with HFIP treatment.
(**A**) Volcano plot of differentially expressed genes with $|Log_2$[Fold Change]$|$> 1 after HFIP treatment compared to LPS treatment only (GSE56256). Down regulated genes were represented by blue dots and up-regulated genes were represented by red dots. (**B**) Heat map of differentially expressed genes after HFIP treatment compared to LPS treatment only (GSE56256). Down regulated genes were marked with blue bars and up-regulated genes were marked with red bars. (**C**) Kyoto Encyclopedia of Genes and Genomes (KEGG) and gene ontology (GO) analysis of up-regulated genes after HFIP treatment in GSE56256. (**D**) KEGG and GO analysis of down-regulated genes after HFIP treatment in GSE56256.

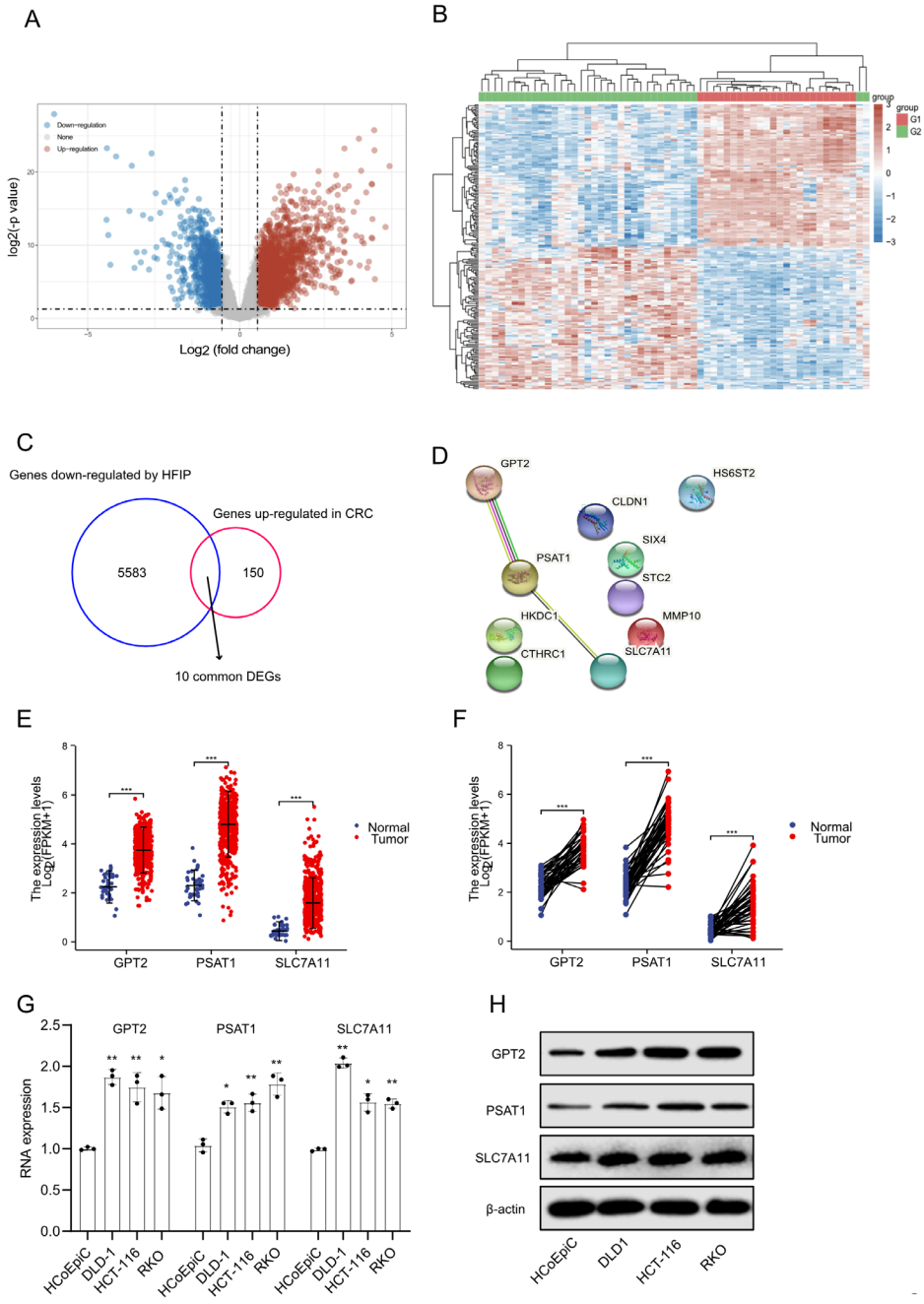

Figure 2. HFIP potentially regulates transaminases in colorectal cancer.
(**A**) Volcano plot of differentially expressed genes in colorectal cancer patients. Down regulated genes were marked with blue dots and up-regulated genes were marked with red dots. (**B**) Heat map of differentially expressed genes in colorectal cancer patients (GSE23878). Down regulated genes were marked with blue bars and up-regulated genes were marked with red bars. (**C**) Venn diagram showed overlapped genes of HFIP down-regulated genes selected from GSE56256 and up-regulated genes in colorectal cancer patients from GSE23878. (**D**) STRING database analysis of selected 10 common DEGs. (**E**) and (**F**) Unpaired and paired analysis of GPT2, PSAT1 and SLC7A11 in TCGA-COAD dataset. (**G**) q-PCR analysis of GPT2, PSAT1 and SLC7A11 in three indicated CRC cell lines (n=3) compared to HCoEpiC cell, normalized to *GAPDH*. (**H**) Western blot analysis of GPT2, PSAT1 and SLC7A11 in three indicated CRC cell lines compared to HcoEpiC cell, normalized to β-actin. The quantification of western blot was also presented (n=3). **P*<0.05, ***P*<0.01, ****P*<0.001.

and gene ontology (GO) analysis. Both KEGG analysis and GO analysis results demonstrated that the up-regulated genes were mainly enriched in pathways and biological processes involving cell cycle, DNA replication (Fig. 1C). For down-regulated genes, they were mainly enriched in chemokine signalling and immune cell functions (Fig. 1D). The above analysis indicates that HFIP treatment reduces inflammation response. Since the chronic inflammation is a significant causal factor in the development of colorectal cancer, our results imply that HFIP has a potential protective effect in colorectal cancer treatment.

Table 2. 10 common DEGs up-regulated in GSE23878 and down-regulated in GSE56256

Gene symbol	Description
STC2	stanniocalcin 2
CLDN1	claudin 1
CTHRC1	collagen triple helix repeat containing 1
PSAT1	phosphoserine aminotransferase 1
HS6ST2	heparan sulfate 6-O-sulfotransferase 2
MMP10	matrix metallopeptidase 10
HKDC1	hexokinase domain containing 1
SLC7A11	solute carrier family 7 member 11
SIX4	SIX homeobox 4
GPT2	glutamic--pyruvic transaminase 2

HFIP potentially regulates transaminases in colorectal cancer

Since inflammation is one of the factors of arising colorectal cancer, we would like to know whether the genes regulated by HFIP in endothelial cells of the lung are also involved in the progression of colorectal cancer. Due to the restricted number of studies performing RNA-Seq in colorectal cancer with HFIP treatment, we selected a colorectal cancer patient cohort (GSE23878) to obtain DEGs that were compared with DEGs from GSE56256. The DEGs from these two GEO datasets were analysed independently. The DEGs with opposite expression patterns in these two GEO datasets may indicate the genes that mediate the function of HFIP, thereby repress the progression of colorectal cancer. GSE23878 contains 35 colorectal cancer samples and 24 normal samples. A total of 150 genes were up-regulated with Log_2[Fold Change] >1 while a total of 610 genes were down-regulated with Log_2[Fold Change] <–1 (Fig. 2A and 2B). Since HFIP is supposed to have a repression effect in cancers, it is intriguing to compare the genes downregulated by HFIP in GSE56256 to the genes up-regulated in colorectal cancer samples in GSE23878. Finally, we obtained 10 genes that overlapped (Fig. 2C) and were listed in Table 2, indicating that these 10 genes may serve as tumor suppressors and mediate the function of HFIP in colorectal cancer. Next, all the 10 genes were analysed *via* STRING database to get the potential protein interaction network. As presented in Fig 2D, only GPT2, PSAT1 and SLC7A11 showed interactions in those 10 genes. GPT2 and PSAT1 are transaminases and SLC7A11 is a cysteine/glutamate transporter (Welch, 1975; Qian *et al.*, 2017; Koppula *et al.*, 2018), which are associated with glutamine metabolism. Furthermore, GPT2, PSAT1 and SLC7A11 contribute to the cancer progression. Interestingly, GPT2, PSAT1 and SLC7A11 were validated to be highly expressed in colorectal cancer from the data of TCGA-COAD dataset (Fig. 2E and 2F). In addition, q-PCR analysis indicated a higher expression level of GPT2, PSAT1 and SLC7A11 in DLD-1, HCT-116 and RKO cells compared to a normal cell line HCoEpiC (Fig. 2G). Western blot also showed similar results (Fig. 2H). Therefore, GPT2, PSAT1 and SLC7A11 serve as oncogenes independent of tissue context. Collectively, HFIP potentially regulates glutamine metabolism in colorectal cancer through mediating GPT2, PSAT1 and SLC7A11.

HFIP represses proliferation of colorectal cancer cells

The effect of HFIP on colorectal cancer cells was investigated in the following studies. The proliferation rate of DLD-1 cells was repressed by HFIP treatment, which was concentration dependent (Fig. 3A). 20 mM HFIP repressed colony formation capability of DLD-

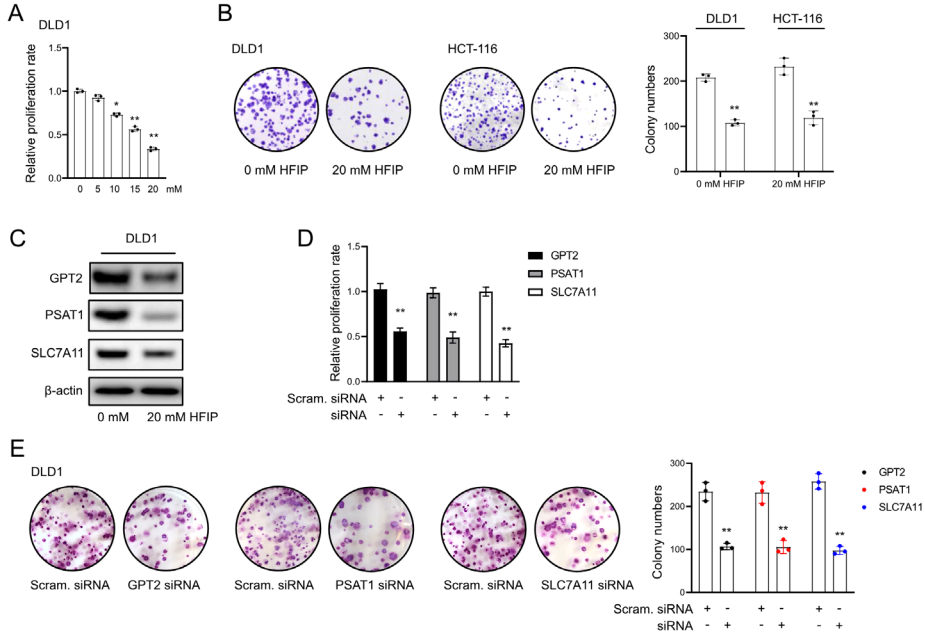

Figure 3. HFIP represses proliferation of colorectal cancer cells.
(**A**) MTT assay showed relative proliferation rate of DLD-1 cells after treated by HFIP with indicated concentration. Proliferation rate was compared to untreated group. (**B**) Colony formation assay was performed in DLD-1 and HCT-116 cells treated with 20 mM HFIP compared to untreated group. (**C**) Western blot analysis of GPT2, PSAT1 and SLC7A11 in DLD-1 cell treated with 20 mM HFIP compared to untreated group. (**D**) MTT assay showed relative proliferation rate of DLD-1 cells after treated by GPT2, PSAT1 or SLC7A11 siRNA. Proliferation rate was compared to scrambled siRNA group. (**E**) Colony formation assay was performed in DLD-1 cell after treated by GPT2, PSAT1 or SLC7A11 siRNA. Proliferation rate was compared to scrambled siRNA group. *P<0.05, **P<0.01.

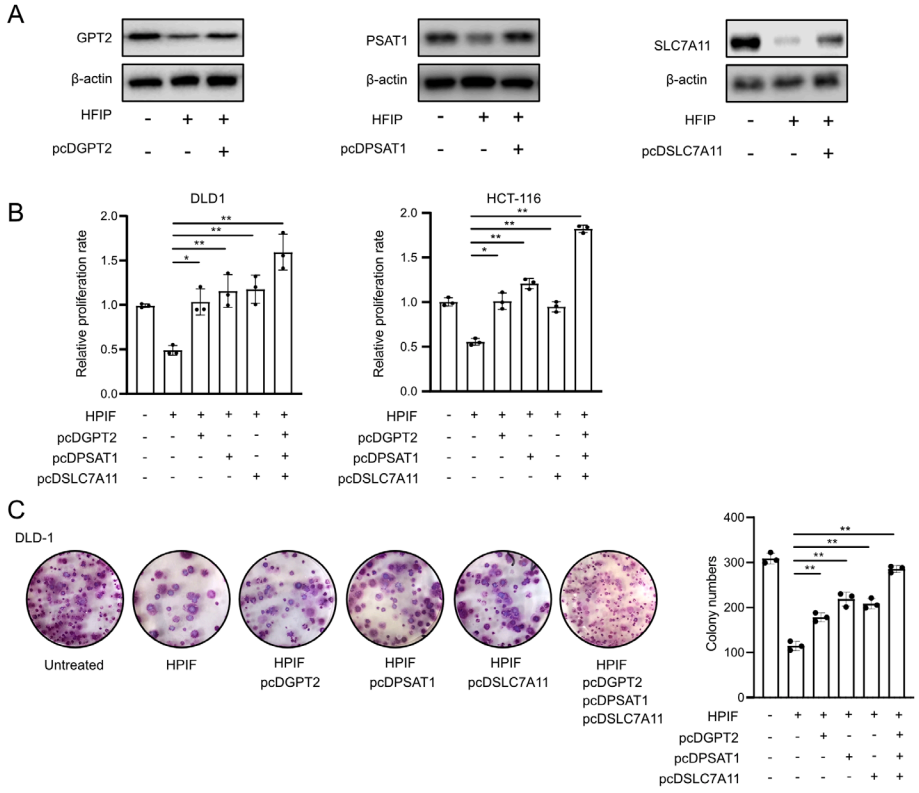

Figure 4. GPT2, PSAT1 and SLC7A11 are required for HFIP effect.
(**A**) Western blot analysis of GPT2, PSAT1 and SLC7A11 in DLD-1 cell treated with 20 mM HFIP together with overexpression vectors. (**B**) MTT assay showed relative proliferation rate of DLD-1 and HCT-116 cells treated with 20 mM HFIP together with overexpression vectors. (**C**) Colony formation assay was performed in DLD-1 cell treated with 20 mM HFIP together with overexpression vectors. **P*<0.05, ***P*<0.01.

1 and HCT-116 cells (Fig. 3B). Above results demonstrated that HFIP suppressed proliferation of colorectal cancer cells. Since GPT2, PSAT1 and SLC7A11 are supposed to be repressed by HFIP in bioinformatic analysis and the expression of these genes was validated in colorectal cancer cells, next we further validated that HFIP treatment significantly repressed the protein level of GPT2, PSAT1 and SLC7A11 compared to control group in DLD-1 cells (Fig. 3C). In addition, silencing GPT2, PSAT1 and SLC7A11 repressed DLD-1 cell proliferation rate (Fig. 3D) and inhibited DLD-1 cell colony formation ability (Fig. 3E). Taken together, HFIP treatment and GPT2/PSAT1/SLC7A11 silence repressed proliferation of colorectal cancer cells, in which the effect of HFIP is potentially mediated by GPT2/PSAT1/SLC7A11.

GPT2, PSAT1 and SLC7A11 are required for HFIP function

As GPT2, PSAT1 and SLC7A11 were validated to be down-regulated by HFIP, here we investigated whether GPT2, PSAT1 and SLC7A11 are required for HFIP function. DLD-1 cells were treated with HFIP and co-transfected with one of the GPT2, PSAT1 and SLC7A11 overexpression vectors. The protein level of GPT2, PSAT1 and SLC7A11 stayed at a high level by co-transfected with overexpression vector and treated with HFIP (Fig. 4A). In both DLD-1 and HCT-116 cells, ectopic expressing GPT2, PSAT1 or SLC7A11 abrogated the effect of HFIP treatment on cell proliferation rate repression. The strongest abrogation effect was observed in the common overexpressing GPT2, PSAT1 and SLC7A11 (Fig. 4B). Similarly, GPT2, PSAT1 or SLC7A11 overexpression abrogated the effect of HFIP on reducing DLD-1 cell colony formation ability (Fig. 4C). Above results indicated that GPT2, PSAT1 and SLC7A11 mediated the effect of HFIP in colorectal cancer cells.

DISCUSSION

In this study, we investigated the function of sevoflurane metabolite HFIP on repressing colorectal cancer cell proliferation. Two transaminases and one cysteine/glutamate transporter were inhibited by HFIP treatment, which mediates the effect of HFIP. Previous studies identified that HFIP is a water soluble primary metabolite of the volatile anaesthetic sevoflurane (Buratti *et al.*, 2002), which is associated with attenuating inflammation through repressing inflammatory mediators (Urner *et al.*, 2015). In line with these conclusions, analysis in RNA-seq data from GSE56256 revealed that HFIP treatment represses more 5000 genes in LPS induced inflammatory response cases. Among those HFIP repressing genes, many genes are mainly enriched in signalling pathway and biological process involving cytokine-cytokine receptor interaction and immunocyte proliferation, which provides the evidence that HFIP has an effect on inflammation.

Since inflammation plays a pivotal role in tumorigenesis *via* multiple molecular mechanisms, here we explored the effect of HFIP on colorectal cancer, which is at a high risk for the patients with inflammatory bowel disease (Herszenyi *et al.*, 2015). Since few studies performed Micro-array analysis in colorectal cancer patients

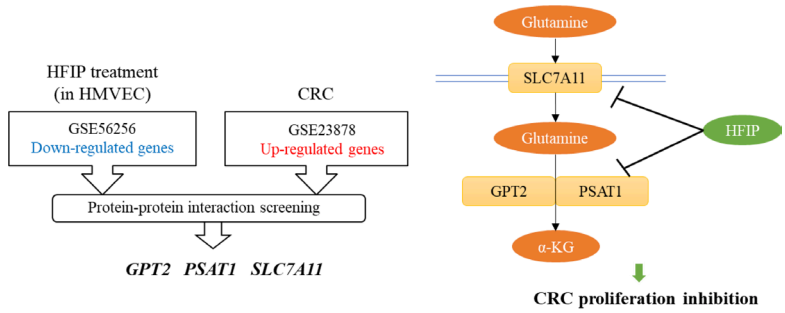

Figure 5. The regulation model of HFIP in colorectal cancer.
GPT2, PSAT1 and SLC7A11 were screened from two independent patient cohorts. HFIP repressed proliferation of colorectal cancer cells through down-regulating GPT2, PSAT1 and SLC7A11 expression, which are involved in glutamine metabolism of colorectal cancer.

with HFIP treatment, we endeavoured an alternative way to find the tumor suppressive genes that are involved in the function of HFIP: We performed differentially expressed gene analysis in a colorectal cancer patient cohort (GSE23878) to obtain DEGs that were compared with DEGs from GSE56256. The DEGs from these two GEO datasets were analysed independently. The DEGs with opposite expression patterns in these two GEO datasets indicate the genes that mediate the function of HFIP, thereby repress the progression of CRC. We obtained 10 up-regulated genes in GSE23878 (CRC patient cohort) that were also down-regulated in GSE56256 (HFIP cohort). Since the two GEO datasets are from different tissues, we exclude the context specificity of the 10 genes. Among those 10 genes, GPT2, PSAT1 and SLC7A11 showed connections with each other, which implies that these three genes play critical roles as a network in HFIP function. Actually, GPT2, PSAT1 and SLC7A11 are involved in glutamine metabolism in cancer progression dependent on proliferation and cell growth (Cao *et al.*, 2017; Qian *et al.*, 2017; Koppula *et al.*, 2018). Indeed, altered cellular energetics is an established hallmark of cancer. Glutamine normally provides carbon and nitrogen for biosynthesis involved in cancer survival and proliferation. SLC7A11 functions as a cysteine/glutamate transporter, while GPT2 and PSAT1 are transaminases that convert glutamine to AKG. Here we confirmed that HFIP treatment repressed proliferation of colorectal cancer cells. Meanwhile, silencing GPT2, PSAT1 and SLC7A11 also inhibited cell proliferation. Therefore, we suppose that the effect of HFIP on cell proliferation repression is mediated by GPT2, PSAT1 and SLC7A11. In addition, glutamine can be transported into cells by the SLC7A11, and it is possible that SLC7A11 takes first place in the response to HFIP treatment.

Furthermore, Overexpression of GPT2, PSAT1 and SLC7A11 abrogated the effect of HFIP on repressing colorectal cancer cells, which demonstrates that GPT2, PSAT1 and SLC7A11 are necessary for HFIP function. Notably, common overexpressing of GPT2/PSAT1/SLC7A11 showed the strongest effect on the abrogation of HFIP function. Therefore, GPT2, PSAT1 and SLC7A11 may have a synergetic effect and mediate HFIP effect together.

In conclusion, HFIP represses proliferation of colorectal cancer cells through down-regulating GPT2, PSAT1 and SLC7A11 expression, which are involved in glutamine metabolism of colorectal cancer, summarized in Fig. 5.

Declarations

Data availability. The authors confirm that the data supporting the findings of this study are available within the article.

Conflicts of Interest. The authors declare that there is no conflict of interest regarding the publication of this article.

Acknowledgement. None.

Funding statement. Do not apply.

Author contributions statement. Z.X.S: conception and design of study, acquisition of data, analysis and/or interpretation of data, drafting the manuscript. This author is co-first author. L.S.: conception and design of study, acquisition of data, analysis and/or interpretation of data, drafting the manuscript. This author is co-first author. W.X.L: conception and design of study, revising the manuscript critically for important intellectual content supervising the study.

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