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FULL LENGTH ARTICLE

Depression and stress levels increase risk of liver cancer through epigenetic downregulation of hypocretin

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Abstract Recent studies suggest that Hypocretin (HCRT, Orexin) are involved in stress regulation of depression through the hypothalamic-pituitary-adrenal (HPA) axis. However, the molecular mechanism by which Hypocretin regulate neurobiological responses is unknown. Herein, the effects of chronic stress on the epigenetic modification of HCRT and its association with depression were explored with regard to a potential role in cancer progression. In the study, Sprague Dawley (SD) rats were used to establish an animal model of cancer with depression by administrating n-nitrosodiethylamine (DEN) and chronic unpredictable mild stress (CUMS). RNA-sequencing was used to detect differentially expressed genes in the hippocampus of rats and quantitative real-time polymerase chain reaction (qRT-PCR) was used to validate the results of RNA-sequencing. The status of HCRT promoter methylation was assessed by methylation specific polymerase chain reaction. Behavioral tests showed that rats exposed

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to CUMS had significant depressive-like behaviors. The number of liver tumors and tumor load in depressed rats exposed to CUMS was higher than in SD rats without CUMS. RNA-sequencing revealed that HCRT was one of the most siginificantly downregulated gene in the hippocampus of SD rats with CUMS compared to non-stressed group, which was validated by qRT-PCR. HCRT mRNA expression was downregulated and the promoter for HCRT was hyper-methylated in those with depression. These results identified a critical role for chronic psychological stressors in tumorigenesis and cancer progression, via epigenetic HCRT downregulation. Such epigenetic downregulation may be the molecular basis for the association of cancer with depression.

Introduction

Major depressive disorder (MDD) is a serious mental disorder, prevalently among cancer patients. Among cancer patients, the morbidity of depression is approximately 12.5%, which is four times that of the general population.[1] Epidemiological studies have found that depression have a lot of negative influence on cancer populations, including shortened survival,[2,3] fatigue, reduced treatment compliance, reduced quality of life,[4] and increased risk for suicide.[5] Depression has been associated with poor prognosis and increased cancer mortality.[6] A meta-analysis of 160,000 patients found that self-report psychological stress is epidemiologically associated with multiple cancer deaths.[7] However, cancer is a highly heterogeneous disease, cancer occurrence and development affected by a variety of factors, and the relationship between depression and cancer is still remind controversial. In recent years, advances in the neurobiology of depression and the physiopathology of cancer have identified common bio-behavioral mechanisms suggested associations between depression and cancer progression. Chronic stress-mediated inflammatory dysbiosis and immunosuppression increased susceptibility for many chronic diseases, including depression and cancer.[8–10] Stress appears to contributed to different stages of tumor progression, including tumorigenesis, angiogenesis and diffusion metastasis.[11] At the molecular level, epigenetic regulation influences gene expression in susceptible individuals, and which may be associated with the interactions among environmental stress exposure, endocrine modulation and immune dysfunction.[12]

Hypocretin (HCRT, also known as Orexin)[13] is an excitatory neuropeptide secreted by neurons on the lateral side of the hypothalamus and around the fornix,[14] which acts via two G protein-coupled receptors, hypocretin-receptor-1 and -2.[15] HCRT and its receptors are found in many brain regions including the prefrontal cortex (PFC), hippocampus, almond nucleus and ventral tegmental area.[16–19] These regions are closely associated with the hypothalamic-pituitary-adrenal (HPA) axis, which controls the response to stress,[20–22] and are involved in the homeostasis of digestion, the immune system, emotion, cognition,[23,24] and energy metabolism.[25,26] Many preclinical studies have shown that depressive-like behavior in rodents is associated with the function of HCRT.[27–30] Interestingly, the enhanced or weakened function of HCRT is not only related to the type, intensity, duration, coping style of stress and the response to stressors,[31,32] but also to different functions of various projected brain regions.[33] However, the cause and the underlying basis for these differences are unclear. DNA methylation[34] is an important epigenetic mechanism that promotes tumorigenesis and tumor progression by silencing tumor suppressor genes.[35] Recent evidences have demonstrated that DNA methylation seems to be associated with depression.[36–38]

In order to explore the effect of depression on cancer and the molecular basis for their association, we developed a rat model of hepatocellular carcinoma (HCC), also with depression. In this study, we used this rat model to explore the function of HCRT in the hippocampus, firstly revealed that the epigenetic modification of HCRT plays a crucial role in the association between depression and HCC progression.

Materials and methods

Animal study

Thirty-four Sprague Dawley (SD) male (80—100 g, ~3 weeks of age) rats were purchased from Experimental Animal Center of Chongqing Medical University (Chongqing, China). Single-housed rats were kept in animal room at 21 ± 1 °C under, a 12 h dark/light cycle (lights on from 7:00 a.m. to 7:00 p.m.) and 50 ± 5% humidity., Food and water were provided *ad libitum*. Animal use and procedures were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Ethics Committee of Chongqing Medical University, Chongqing, China. After 1 week of acclimation, the SD rats were weight-matched and randomly divided into three groups: Normal control group (Control, $n = 8$), Hepatocellular carcinoma group (HCC, $n = 10$), and HCC with Depression group (HCC+ Dep, $n = 16$). Rats in the HCC group were administered with a single intraperitoneal injection of n-nitrosodiethylamine (DEN) at a dose of 25 mg/kg of body weight dissolved in 0.9% saline, and started to drink water with 0.01% DEN continuously after 1 week of DEN injection.[39,40] Besides administered with DEN, Rats in the HCC with Depression group were repeatedly exposed to two or three kinds of chronic unpredictable mild stressors at random each day, which including: food or water

deprivation, damp bedding (300 mL of water spilled per cage), 45° cage-tilt along the vertical axis, turning night into day, stroboscopic illumination (300 flashes/min) sleep deprivation, behavior restriction, 4 °C ice water swimming and tail nip. The same stimulus was not repeated in two days. Normal control rats were given i.p. injections of saline and normal drinking water without CUMS. After 17 weeks of HCC induction, SD rats were tested for behaviors. For the pre-experiment section, CUMS last two months was used to induce depression-like behavior in C57 mice. Mice were divided into two groups, depression group and control group, behavioral tests were done according to the methods reported in the literature[41] and tissue samples were collected.

Behavioral tests

Sucrose preference test

In the sucrose preference test (SPT),[43] the rats were been deprived food and water for 12 h before test began. Two transparent water bottles of the same size and shape, one been filled with clean water and the other with 1% sucrose solution, were provided to the same rat concurrently. The test lasted 1 h and bottles with water or solution been weighted before and after the test. The proportion of sucrose consumption in total consumption was calculated.

Open field test

Each rat was placed in the center of an open cube box (100 × 100 × 40 cm) with black opaque bottom and peripheral wall, the movements within 5 min were recorded by a video surveillance (logitech webcam software), and rats allowed 30 s to adapt to the observation box.[42] The total distance travelled for 5 min and the time spent in central zone was assessed. The open-field test (OFT) was carried out in a sound-attenuated room with rats separated to avoid communication between each other. This test been measured one day before modeling and after 17 weeks of CUMS treatment.

Tissue sample collection

Rats were sacrificed the day after the behavioral tests were completed. Hippocampus and liver tissue were immediately dissected, frozen in liquid nitrogen and stored at −80 °C.

DNA and RNA extraction

Genomic DNA was extracted from tissue and cell samples using DNAzol® Reagent (Invitrogen) and the QIAamp® DNA Mini Kit (Qiagen). Total RNA was isolated from tissue and cell samples using TRIzol® (Invitrogen). The concentration of RNA was determined by spectrophotometry using NanoDrop™ 2000 (Thermo Scientific). DNA and RNA integrity were determined using gel electrophoresis.

Hematoxylin and eosin (HE) staining

Liver tissue was fixed in 10% neutral buffered formalin and embedded in paraffin. After dewaxing and rehydration, HE staining was performed on 5-μm sections following a standard protocol.

Immunohistochemistry

Immunohistochemistry was executed using the UltraSensitive TM SP kit (Maixin-Bio, Fujian, China) according to the instructions. Ki-67 (#9449, Cell Signaling Technology) and Glypican-3 (#MAB-0617, Maixin-Bio, Fujian, China) are diluted at recommended concentrations. The results were determined according to previously published methods.[44]

RNA-sequencing

RNA samples were sent to a Genomics facility (Novogene, Beijing, China) for RNA-Seq. The quality of RNA was assessed using a NanoPhotometer spectrophotometer and a 2100 Bioanalyzer instrument. An RNA library was prepared using the NEB Next Ultra Directional RNA Library Prep Kit for Illumina. Poly-A mRNA was enriched with Oligo (dT) magnetic beads. Libraries were sequenced using an Illumina HiSeq 2000.

Semi-quantitative polymerase chain reaction (PCR) and quantitative real-time PCR (qRT-PCR) analysis

Reverse transcription was performed using Promega GoScript™ reverse transcriptase (Promega). Two μL of cDNA (1200 ng/μL) were added to a 10 μL RT-PCR reaction mixture. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the control. The primer sequences and specific conditions are listed in Table 1. PCR was performed using Go-Taq (Promega). Gel electrophoresis (120 V, 25 min) was performed using 2% agarose gels. Results were obtained using a BioRad Gel Doc XR + system. The qRT-PCR was performed using SYBR Green (Thermo Fisher) following the manufacturer's instructions (7500 Real-Time PCR System, Applied Biosystems, Foster City, CA, USA). The primers are listed in Table 1. Each sample was tested in triplicate. Gene expression levels were calculated by the $2^{-\Delta\Delta Ct}$ method.[45]

Methylation-specific PCR (MSP)

Methylation specific polymerase chain reaction (MSP) was conducted for 40 amplification cycles using AmpliTaq®-Gold DNA polymerase (Applied Biosystems), with annealing temperatures of 60 °C and 58 °C for methylated and unmethylated samples, respectively (Table 1). Amplicons were analyzed as previously described.[46]

Clinical research

Subject recruitment

All subjects were recruited from the First Affiliated Hospital of Chongqing Medical University. This study was approved by the Institutional Review Committee and informed consent obtained from all participants. Demographic and clinical characteristics of the participants were collected in the form of a questionnaire including information such as sex, age, body mass index (BMI), tumor type, disease stage, treatment, family history of mental illness, and drug abuse. Individuals who reported current alcohol/substance dependence, lifetime psychotic or bipolar disorder, a family history of MDD, bipolar disorder, or suicide were excluded.

Table 1 List of primers used in this study.

PCR	Primer	Sequence (5′—3′)	Product size (bp)	PCR cycles	Annealing temperature (°C)
	rHcrt-F	TGACGCTGCTGCTGCTGCTA			
	rHcrt-R	TCGCCGCTTTCCCAGAGTGA	170		60
	HCRT-F	CGTGACGCTACTGCTGCT			
	HCRT-R	GTGCAGCAGCTCGTAGAGG	128		60
	rKi67F	CTGACCCTGATGGGAAAGAT			
	rKi67R	TGCGATCGTGCTGTTCTACA	165		60
	rSurvivinF	TTAAGGAACTGGAAGGCTGG			
	rSurvivinR	TCAGTTCTTCCACCTGCTTC	108		60
	rOx1rF	AGGGAATACCTACACCCGAA			
	rOx1rR	AGTTGGTGACTGTCCTCATG	139		60
	rOx2rF	CACATGAGGACAGTCACCAA			
	rOx2rR	GTGATGTCCACTAACAGGCT	101		60
MSP	rHcrt-m1	TTGTCGTCGGCGTTGTTGTC			
	rHcrt-m2	CAAAATAAAAATACCCGCGACG	135	40	60
	rHcrt-u1	TTGTTGTTGTTGGTGTTGTTGTT			
	rHcrt-u2	CCAAAATAAAAATACCCACAACA	139	40	58
	HCRT-m1	CGTTGTTGTCGTTCGGGGC			
	HCRT-m2	AACGTAAAAATACCGACCGCG	124	40	60
	HCRT-u1	TGTGTTGTTGTTGTTTGGGGT			
	HCRT-u2	CCAACATAAAAATACCAACCACA	128	40	58

qRT-PCR: Quantitative reverse transcription-PCR; MSP: Methylation-specific PCR.

Diagnosis and assessment of depression

Based on the criteria of the American Diagnostic and Statistical Manual of Mental Disorders V (DSM-V), Hamilton depression scale (HAMD-17)[47] was used to evaluate depression in patients with cancer who met the inclusion criteria. The severity of depression was graded by the HAMD score.

Whole blood sample determination

After giving written informed consent, venous blood collection was performed. Leukocytes were isolated from whole blood. RNA and DNA were extracted for qRT-PCR and MSP, respectively.

Statistical analysis

SPSS 20.0 was used to perform statistical analysis. Difference between two groups was tested by χ^2. The difference among groups was assessed by one-way ANOVA. Data are expressed as means ± SEM. A *P*-value less than 0.05 was considered statistically significant.

Results

CUMS and HCC animals

CUMS induced depression-like behavior in HCC rats

Weight gain for rats exposed to CUMS was reduced (Fig. 1A). For the SPT, HCC + Dep rats showed obvious depressive-like behavior, and were found to have lower sucrose preference (%) than that of control ($P < 0.05$). The preference for sucrose was significantly different for HCC *vs*. HCC + Dep ($P < 0.05$) (Fig. 1B). For the OFT, the locomotor activity of rats for CUMS was decreased, with obvious anxiety-like behavior. Rats in HCC + Dep traveled a shorter distances than control ($P < 0.05$), and the total traveling distance for 5 min was significantly increased for HCC *vs*. HCC + Dep ($P < 0.05$) (Fig. 1C). The time rats in HCC + Dep spent in central zone was significantly decreased ($P < 0.05$) (Fig. 1D). OFT, SPT and forced swimming test (FST) was performed in C57 mice, the locomotor activity has no statistical difference, but lower sucrose preference and longer immobility time was been observed in mice with CUMS (Fig. S1A—C).

Effect of CUMS on the tumorigenesis of liver in rats

After 17 weeks of DEN induction, the survival rates of HCC + Dep group and HCC group were 81% (13/16) and 100% (10/10) respectively, the tumor formation rate of HCC + Dep group was 100% (13/13), and that of HCC was 80% (8/10). Rats was scattered, multiple nodules were seen in the liver of rats. Tumor burden of HCC + Dep rats was significantly greater than HCC rats (Fig. 2A). The liver tissue is weighed, nodules larger than 1 mm in diameter on the liver surface were counted and measured. The number and the size of the tumors in HCC rats were significantly lower than those in HCC + Dep rats, the liver/body weight ratios in HCC rats with or without CUMS were both higher than that in control rats (Fig. 2B). The hepatic lobular structure of HCC rats with or without depression was seriously destroyed, and heterotypic cells and cancer cells of different size were seen under microscope (Fig. 2C). Metastatic cancer cells were not be found in lung tissue of HCC rats with or without depression (Fig. 2D).

Compared with the control group, biochemical indicators ALT and AST both were significantly increased in HCC rats with or without depression (Fig. 3A). The

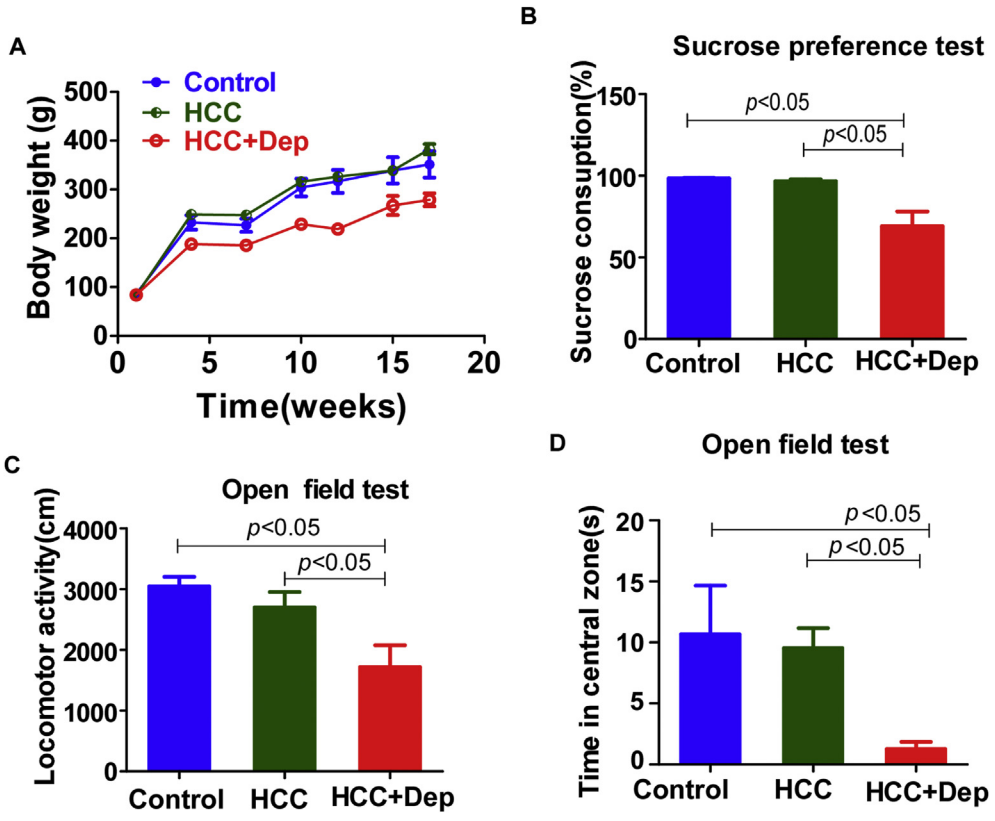

Figure 1 Behavioral tests in SD rats after the final HCC induction administration. **(A)** Results of weight gain trend in rats within 17 weeks. **(B)** Results of percentage of sucrose consumption in the sucrose preference test. **(C)** Results of the locomotor activity (cm) was recorded for a 5-min test session in the open field test. **(D)** Results of the time spent in central zone was observed for a 5-min test session in the open field test. Data are expressed as mean ± SEM. Statistical analysis was performed by one-way ANOVA. Compared with control, $^{*}P < 0.05$.

expression of Ki67 or Survivin, tumorigenesis related biological markers, was increased in HCC *vs*. control, and also increased in HCC + Dep *vs*. HCC (Fig. 3B). Tumorigenesis at the protein level was further validated by immunohistochemistry (IHC) staining. Stronger nuclear staining of Ki67 and stronger plasma staining of Glypican 3 were observed in HCC + Dep than that in HCC (Fig. 3C, D).

RNA-sequencing

Differentially expressed genes (DEG) within the hippocampus of HCC Rats with or without depression was assessed. A total of 339 DEGs were identified in HCC + Dep *vs*. HCC, including 182 upregulated and 157 downregulated genes (Fig. 4A). Of the 16,099 genes assessed, 14,357 were identified in HCC and in the HCC + Dep groups (Fig. 4B). The clustering of DEGs in different samples showed that Hcrt was downregulated in HCC + Dep *vs*. HCC (Fig. 4C). DEGs within the hippocampus of C57 mice with or without depression was been observed, 1210 upregulated and 922 downregulated genes was obtained separately of total 23,524 identified DEGs (Fig. S1D). The 21 enriched pathways revealed by KEGG analysis in Depression *vs*. Control are shown as a Bubble Diagram, this analysis revealed enriched pathways to be mainly concentrated in neuroactive ligand—receptor interaction, calcium signaling pathway, axon guidance, and ras signaling pathway (Fig. S1E). The clustering of DEGs between two groups revealed that Hcrt was downregulated in Depression *vs*. Control (Fig. S1F).

RNA-Seq was used for three pairwise comparisons of DEGs in liver tissue of rats, Control *vs*. HCC, Control *vs*. HCC + Dep, and HCC *vs*. HCC + Dep. Volcano plots (Fig. 5A) showed the distribution of DEGs for each comparison. The heat map (Fig. 5B) provided a visual representation of expression levels for all DEGs of the three groups. The Venn diagram (Fig. 5C) describes the total number of DEGs in each pairwise comparison and shows the number of shared DEGs between different comparisons. The results showed that HCRT was significantly down regulated both in rat's hippocampus and liver in the HCC with Depression group compared to the other two groups.

Epigenetic Hcrt downregulation is associated with emotional regulation in rats

To validate RNA-Seq results, the hippocampal expression of Hcrt in HCC and HCC + Dep was assessed. Compared to HCC, Hcrt expression was significantly lower in the hippocampus of HCC + Dep rats (Fig. 6A). Results were further

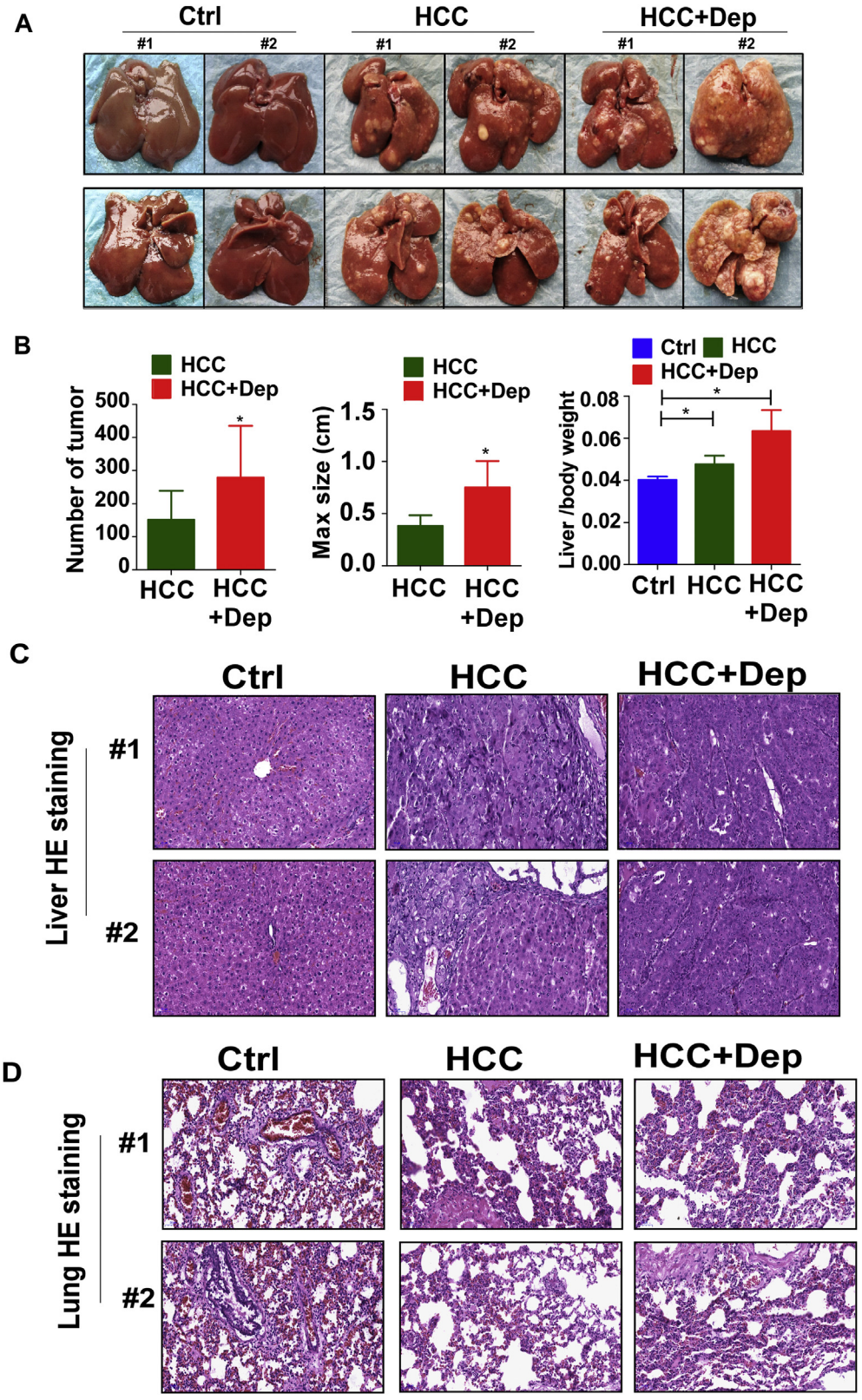

Figure 2 Positive effect of CUMS on the tumor burden of the liver in rats. **(A)** Representative images with front and back side of liver from three groups rats (Normal control, HCC and HCC + Dep rats). **(B)** Results of tumors counts on the liver surface, max tumor size and the rate of liver weight/body weight. Results are shown as the mean ± SEM, $^*P < 0.05$. **(C, D)** Representative H&E staining of liver and lung sections in three groups of rats. Scale bars 50 μm.

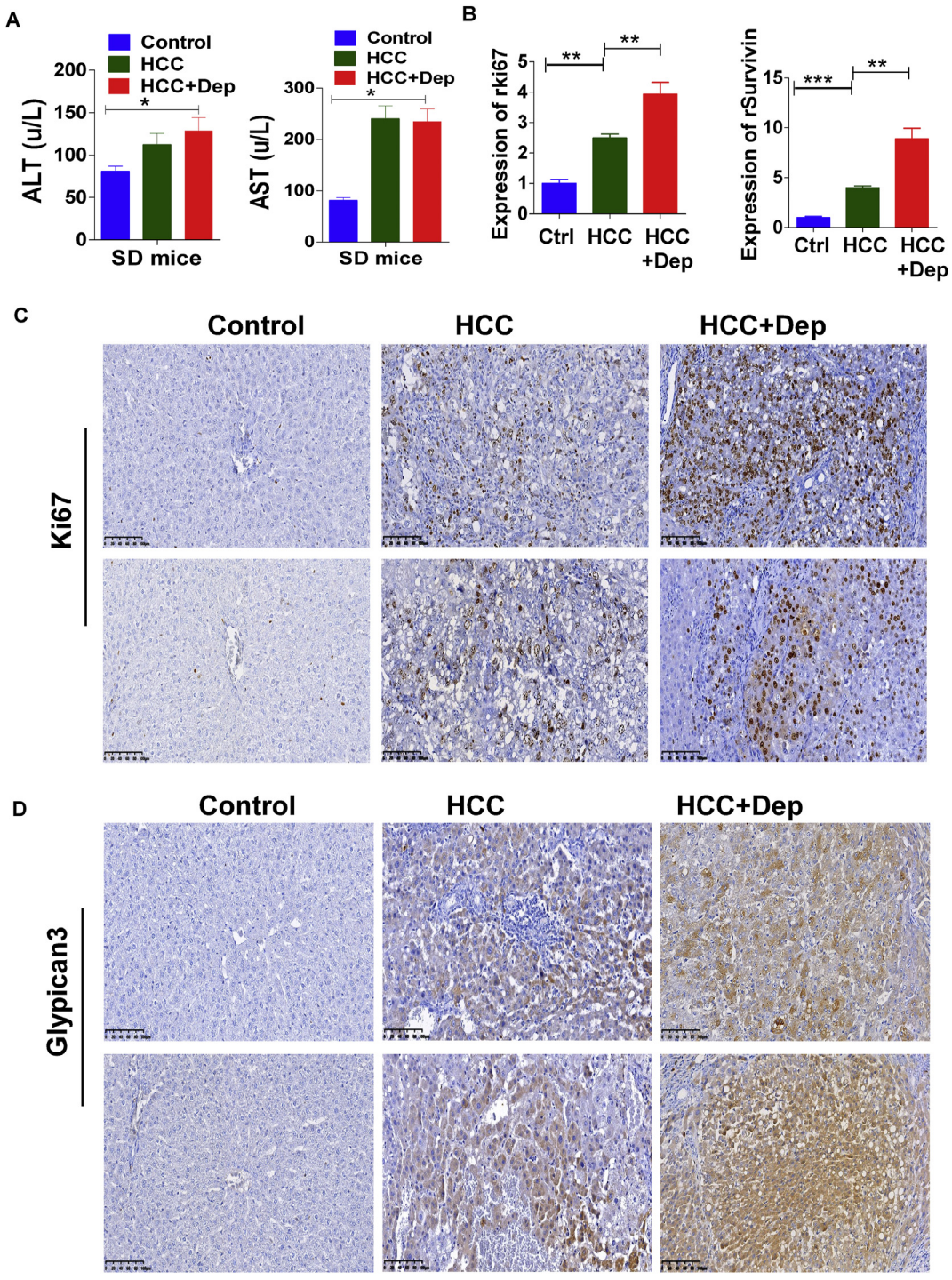

Figure 3 Positive effect of CUMS on the tumorigenesis and progression of the liver in rats. **(A)** Results of liver function in three groups of rats. ALT, Alanine transaminase; AST, aspartate aminotransferase. Data represent mean ± SEM, $^{*}P < 0.05$. **(B)** The expression of Ki67 mRNA and Survivin mRNA were detected by qRT-PCR ($^{**}P < 0.01$, $^{***}P < 0.001$). **(C, D)** Validation of tumour tumorigenesis at the protein levels by IHC staining. Scale bars 100 μm.

validated by analysis of Hcrt expression in the livers of three groups of rats (Fig. 6B,F). Hcrt mRNA expression was significantly reduced in HCC *vs*. Control, and further reduced in HCC + Dep *vs*. Control. The mRNA expression of orexin receptor 1(Ox1r) and orexin receptor 2 (Ox2r) in the hippocampus of HCC rats were detected, the expression of Ox1r mRNA has no significant difference in the comparison of HCC + Dep *vs*. HCC, but Ox2r mRNA expression was significantly reduced in HCC + Dep *vs*. HCC (Fig. 6C). Subsequently, Ox2r mRNA expression was detected to be down-regulated in HCC and further down-regulated in HCC + Dep compared with control group (Fig. 6D). HCRT mRNA

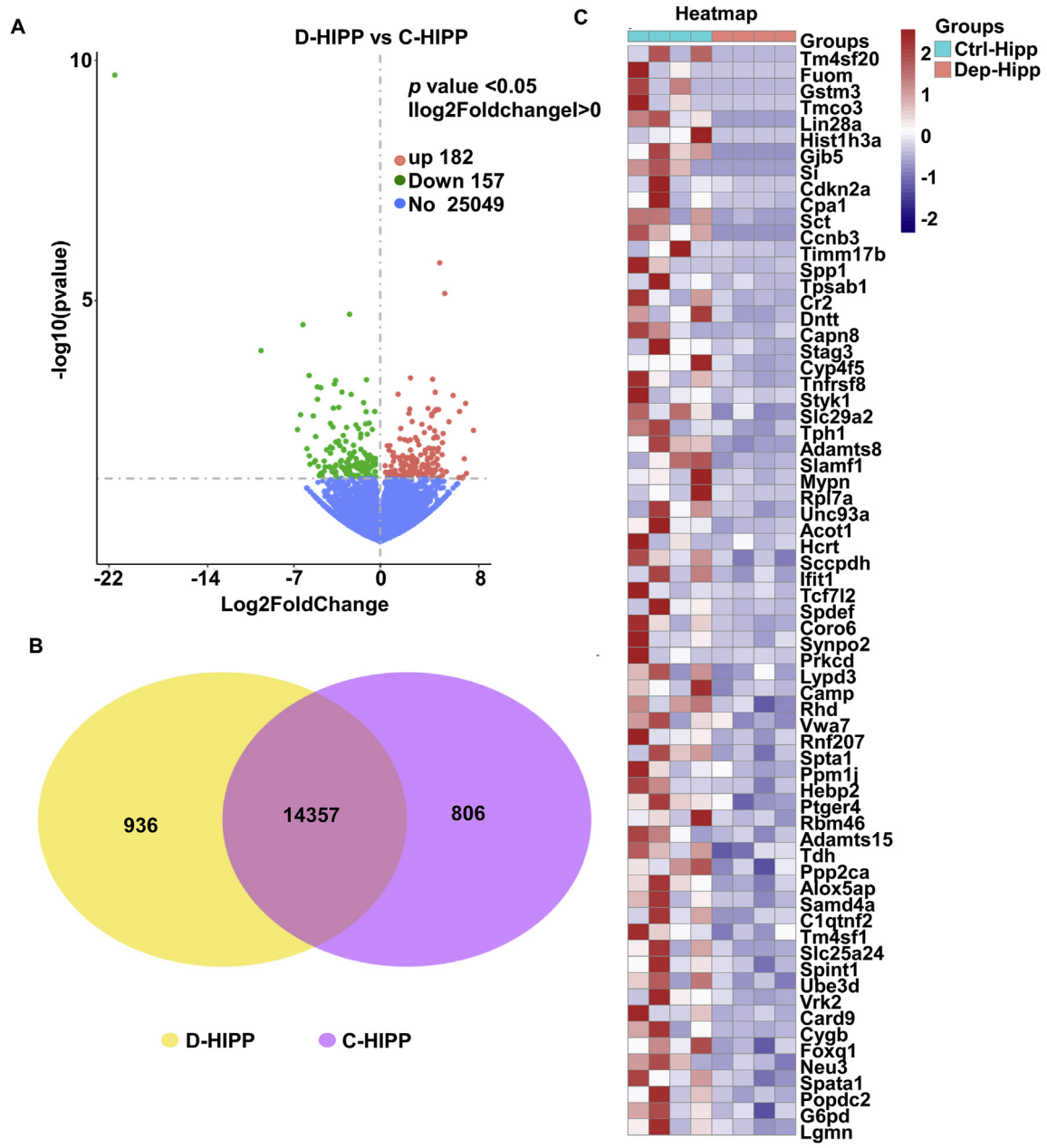

Figure 4 Visual summary of DEGs of hippocampus in HCC rats between depression and control groups. **(A)** Volcano plots highlighting DEGs in each comparison by plotting$-\log_{10}$(*P*-value) against $\log_2$(Fold-change) of individual genes. **(B)** Venn diagram illustrating the number of shared genes between each comparison. **(C)** Heatmap showing the clustering analysis of DEGs among different samples. Red color indicates high gene expression; blue color indicates low gene expression.

expression in whole blood tissues of human was been detected, the result indicated that HCRT down-regulated in patients with depression compared with normal (Fig. 6E). These results are consistent with RNA-Seq data, suggesting a crucial role for Hcrt in rat emotional regulation.

To investigate whether promoter hyper-methylation was responsible for decreased Hcrt expression in CUMS rats, DNA samples from rat hippocampus were assessed by MSP. Higher promoter methylation levels were related to lower Hcrt expression in the hippocampus of CUMS rats (Fig. 6F).

Clinical study of depression in cancer

A total of 123 clinical cancer patients were considered for this study based on the DSM-V diagnostic criteria. Patients were excluded who had primary neurological or psychiatric disorders. Patients were considered to have depression with an HAMD scale score $\geq$17. The demographic characteristics of clinical cases were shown in Table 2. Results showed that there were no significant difference based on age, gender, marital status, stage of tumor, course of disease, type of therapeutic regimen or BMI in comparison of

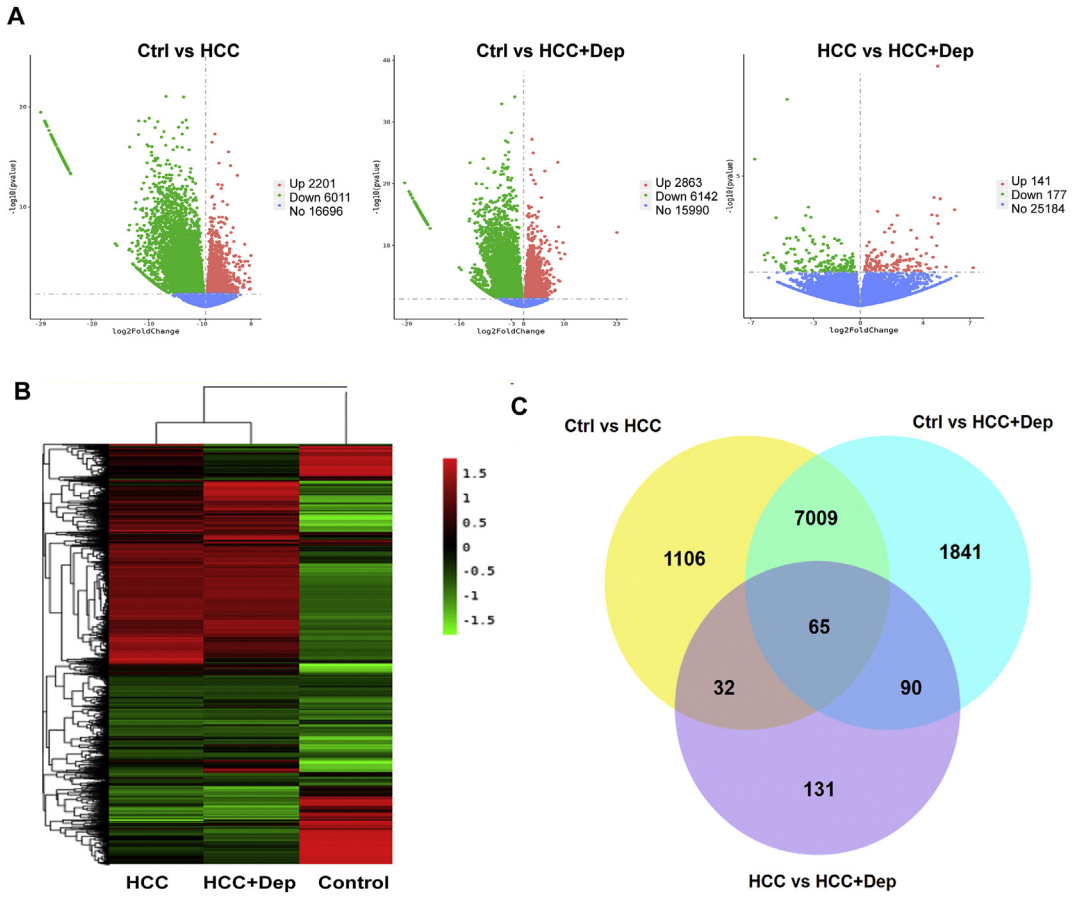

Figure 5 Visual summary of DEGs of liver tissues in rats between all three groups. **(A)** Volcano plots showing the DEGs for three pairwise comparisons, red dots for upregulated genes, green dots for downregulated genes. **(B)** Heatmap showing the level of expression [in $\log_{10}$(FPKM+1)] for all DEGs of three groups. FPKM, expected number of Fragments Per Kilobase of transcript sequence per millions base pairs sequenced. **(C)** Venn diagram describing the overlap of DEGs between three pairwise comparisons. Overlap regions, the number of differential genes shared to the different combinations.

cancer patients with or without depression ($P > 0.05$). Among cancer types, lung cancer, colorectal cancer, breast cancer and nasopharyngeal carcinoma accounted for a higher proportion than others. No statistical difference was found in the comparison of cancer types between the two groups ($P > 0.05$).

Promoter methylation downregulates HCRT in patients with depression

To confirm whether promoter hypermethylation was responsible for decreased HCRT expression in depression, blood leucocyte DNA from clinical patients was assessed by MSP. The HCRT promoter was methylated in 25 of 41 (61%) cancer patients with depression and methylated in 7 of 23 (30%) of cancer patients without depression. Moreover, the HCRT promoter was essentially unmethylated in 16 normal control blood samples, and methylated in 3 of 7 (43%) blood samples from individuals with simple depression (Table 3). In summary, greater promoter methylation levels were associated with lower HCRT expression and depression (Fig. 7A–C). Overall survival was further explored between HCRT high methylation and HCRT low methylation, and it turns out that high methylation of HCRT is associated with lower survival rates ($P < 0.05$) (Fig. 7D).

Discussion

In this study, we provide evidence that supports HCRT as an emotional regulatory factor that influences the affective behavioral phenotype, depression. The stress-induced depressive phenotype promoted tumorigenesis and progression of HCC. HCRT expression was downregulated in depression and HCC, which has been shown to be associated with HCRT promoter methylation. Further, epigenetic HCRT downregulation was more significantly in HCC with depression, and higher promoter methylation levels was related to lower HCRT expression in those with depression. These results suggested epigenetic HCRT promoter methylation may be a molecular mechanism by which depression is associated with cancer.

Previous studies have found Hcrt to be associated with depressive-like behaviors in rodents.[27–30] This is consistent with the results of this study in that rats exposed to CUMS for an extended period had significant anxiety and

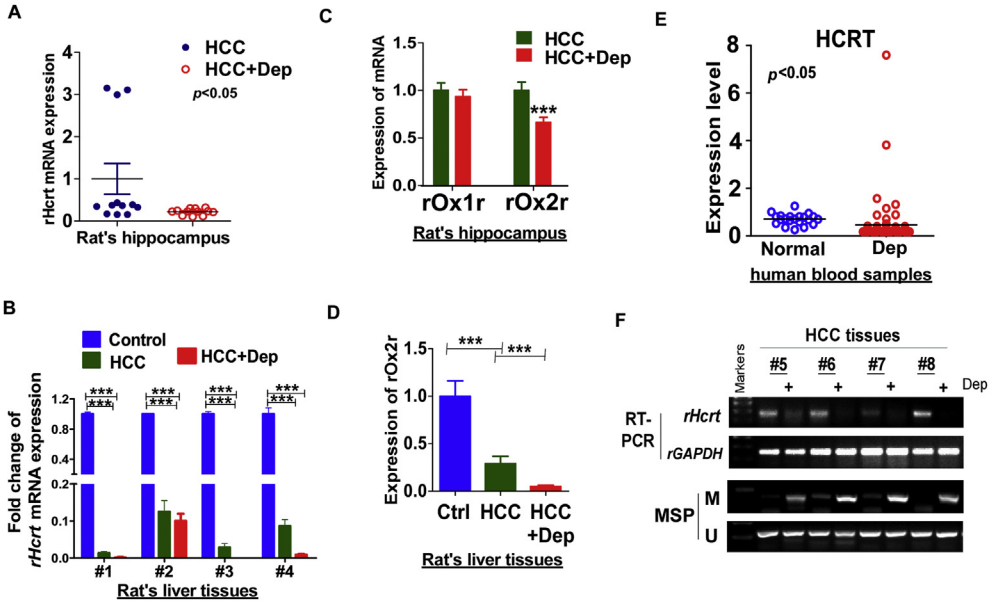

Figure 6 Expression and methylation of Hcrt and it's receptors in rats and human. **(A)** Hcrt mRNA expression in hippocampus of HCC rats and HCC + Dep rats detected by qRT-PCR (*$P < 0.05$). **(B)** qRT-PCR analysis of Hcrt mRNA expression in liver tissues of normal rats, HCC rats and HCC + Dep rats (***$P < 0.001$). **(C)** Ox1r and Ox2r mRNA expression in hippocampus of HCC rats and HCC + Dep rats detected by qRT-PCR (***$P < 0.001$). **(D)** qRT-PCR analysis of Ox2r mRNA expression in liver of normal rats, HCC rats and HCC + Dep rats (***$P < 0.001$). **(E)** qRT-PCR analysis of HCRT mRNA expression in blood leucocyte DNA of human (*$P < 0.05$). **(F)** Hcrt expression and promoter methylation in hippocampus of HCC rats and HCC + Dep rats were assayed by RT-PCR and MSP. M, methylated; U, unmethylated. GAPDH was detected as an input control.

depression-like behaviors, with reduced Hcrt mRNA expression in the depressed rats. Interestingly, HCRT function differs in different brain regions,[33] with the function of HCRT especially unclear in the hippocampus. In this study RNA-Seq revealed Hcrt mRNA expression was found to be lower in the hippocampus of depressed mice or rats. Particularly, lower Oxr2 mRNA expression was found in the hippocampus and liver of HCC rats with CUMS than that in HCC rats without CUMS. Our results reveal a crucial role of HCRT in the regulation of depressive behavioral phenotypes, and a recent research evidence provided a reliable support that Orx2 agonist can effectively reduce anxiety and depressive behavior in susceptible mice.[48]

It is well-known that the relationship between depression and cancer initiation and/or progression is very complex. Existing data are inconclusive. Depression has been shown to have negative effects in various cancer populations[2–6] and to increase the risk for cancer.[49] However, other studies showed that depression did not increase the risk for cancer.[50] In the latest preclinical study, social failure rats with long-term anxiety and depression had more lung tumors and higher tumor load.[51] Surprisingly, a similar result was found herein, with the number of liver tumors and tumor burden in rats with CUMS significantly higher than that in rats without CUMS. Expression of Ki67 and Survivin, tumorigenesis related biological markers, were found higher in HCC + Dep group than that in HCC group, suggesting more cell proliferation and less apoptosis occurred in HCC rats with depression. The result was further validated at the protein level. These evidences revealed that long-term psychological stressors contribute to tumorigenesis and cancer progression. In addition, we also found Hcrt to be highly expressed in livers of normal rats, but down-regulated in livers of HCC rats, and even lower in HCC + Dep rats. Similar results were found in whole blood samples from clinic patients. These results suggest that reduced HCRT expression is associated with cancer and depression.

However, the mechanistic basis for the association between cancer and depression remains unclear. Psychological stress-mediated inflammatory dysregulation and immunosuppressive mechanisms are known to increase cancer susceptibility and to influence cancer occurrence, development, and treatment,[51,52] Yet, evidence at the molecular level is lacking. Recent advances in molecular epigenetics have found that epigenetic mechanisms lead to changes in certain biological processes through their long-term effects on gene expression in susceptible individuals.[53,54] Promoter hypermethylation within tumors has been demonstrated by many studies,[55–57] and as well hypermethylation of specific genes has been associated with depression.[58] To explore the molecular basis for the relationship of cancer with depression, we examined the status of HCRT promoter methylation and found promoter

Table 2 Demographic characteristics of cancer patients.

Variables	N = 123 N1 (N1/N ×100)%	Depression n1 = 41 n1 (n1/N1 × 100)%	Non-depression n2 = 82 n2(n2/N1 × 100)%	*P -value*
Age/years	62.0 ± 12.0	64.4 ± 15.1	60.7 ± 10.0	0.107 ▲
≤60	49 (39.8)	12 (24.5)	37 (75.5)	0.090
>60	74 (60.2)	29 (39.2)	45 (60.8)	
Gender				
Male	61 (49.6)	18 (29.5)	43 (70.5)	0.372
Female	62 (50.4)	23 (37.1)	39 (62.9)	
Marital status				
Married	110 (89.4)	35 (31.8)	75 (68.2)	0.300
Unmarried	13 (10.6)	6 (46.2)	7 (53.8)	
Stage of tumor				
I～II	18 (14.6)	4 (22.2)	14 (77.8)	0.279
III～IV	105 (85.4)	37 (35.2)	68 (64.8)	
Course of disease/year				
≤1	80 (65.0)	22 (27.5)	58 (72.5)	0.061
>1	43 (35.0)	19 (44.2)	24 (55.8)	
Type of therapeutic regimen/units				
≤1	54 (43.9)	20 (37.0)	34 (63.0)	0.271
1～2	59 (48.0)	16 (27.1)	43 (72.9)	
>2	10 (8.1)	5 (50.0)	5 (50.0)	
BMI				
<18.5	13 (10.6)	5 (38.5)	8 (61.5)	0.914
18.5～24.99	85 (69.1)	28 (32.9)	57 (67.1)	
>24.99	25 (20.3)	8 (32.0)	17 (68.0)	
Type of cancer				
LC	32 (26.0)	10 (31.3)	22 (68.8)	0.139
CRC	20 (16.3)	8 (40.0)	12 (60.0)	
BRCA	9 (7.3)	6 (66.7)	3 (33.3)	
NPC	21 (17.1)	4 (19.0)	17 (81.0)	
Others	41 (33.3)	13 (31.7)	28 (68.3)	

Note: ▲Results are presented as Mean ± SD; (N: total participants, n1: participants with depression.) LC, lung cancer; CRC, colorectal cancer; BRCA, breast cancer; NPC, nasopharyngeal carcinoma; Others, cervical cancer, esophageal cancer, tonsil cancer, pancreatic cancer, bladder cancer, prostate cancer, gastric cancer, et al.

Table 3 Methylation status of the HCRT promoter in patients with depression.

Samples	HCRT promoter		Frequency of methylation	*P*-value
	Methylated	unmethylated		
Dep ($n = 7$)	3	4	43%	0.000***
Cancer with Dep ($n = 41$)	25	16	61%	
Cancer ($n = 23$)	7	16	30%	
NC ($n = 16$)	0	16	0%	

Dep, depression; NC, normal control; Samples, blood leucocyte DNA of clinical patients. ***$P < 0.001$.

methylation to be greater in those with cancer and depression than in those with cancer but without depression. Furthermore, higher promoter methylation levels were correlated with lower HCRT expression. This study demonstrated HCRT promoter hypermethylation to be a potential molecular mechanism by which cancer is associated with depression. However, further investigation is necessary to elucidate the specific role and function of HCRT in the complex relationship of depression with cancer.

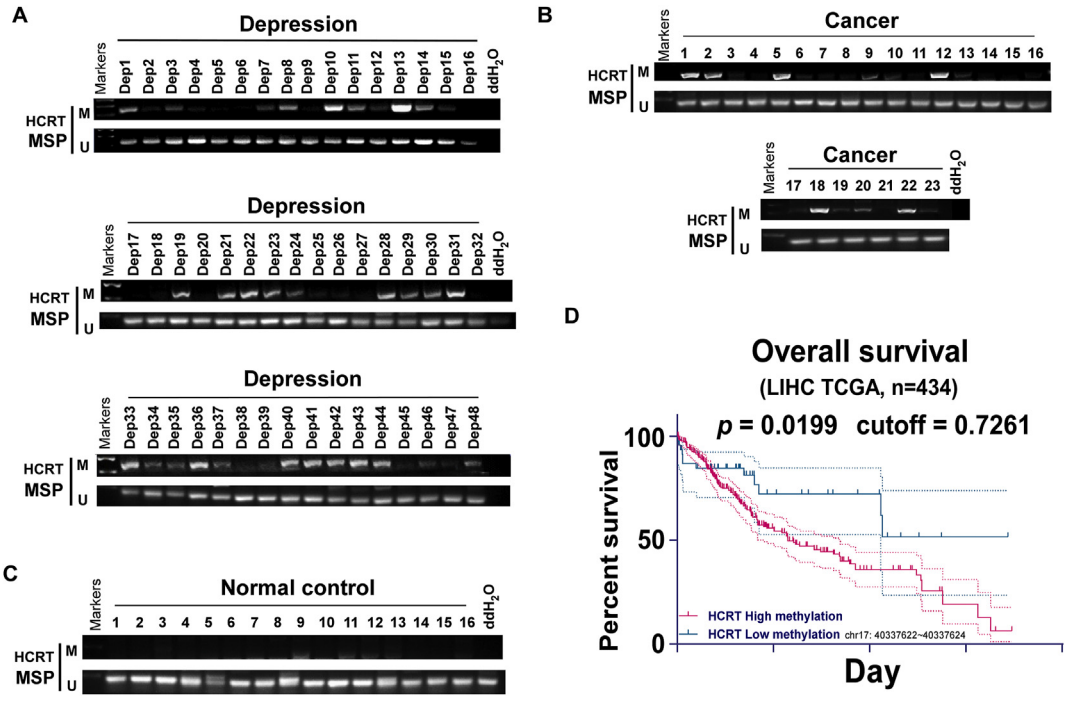

Figure 7 Methylation of HCRT in blood leucocyte DNA of human and the significance of HCRT hypermethylation in the HCC prognostic. **(A)** The methylation status of HCRT in 7 simple depression (Dep1—Dep7) and 41 cancers with depression (Dep8—Dep48) blood samples by MSP. **(B)** The methylation status of HCRT in 23 cancer without depression blood samples by MSP. **(C)** The methylation status of HCRT in 16 normal blood samples by MSP. **(D)** The correlation of hypermethylation of HCRT promoter in patients and the HCC prognostic.

Conclusion

This study demonstrated that psychological stress promoted tumorigenesis and cancer progression. Furthermore, downregulation of HCRT in the hippocampus was shown to play a crucial role in emotional regulation, with epigenetic modification of HCRT expression downregulated in cancer and depression. HCRT promoter methylation may be the molecular basis for the association between depression and cancer progression.

Author contributions

TX: conception and design. CP, ST, SH: performed most experiments. JT, TX: performed experiments and analyzed data. CP, YF,YH,HR,JZ: collected samples. TX, CP,ST: drafted the manuscript. XH, YX: reviewed data and the manuscript. TX: reviewed data and finalized the manuscript. All authors reviewed and approved the final version.

Ethics approval and consent to participate

Informed consent was obtained from each participant included in the study. Our experiments were approved by the Institutional Ethics Committees of the First Affiliated Hospital of Chongqing Medical University [approval notice # 20180307], and the study methodologies conformed to the standards set by the Declaration of Helsinki.

Conflict of interests

The authors declare no conflict of interest.

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Appendix A. Supplementary data

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