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RAPID COMMUNICATION

ARIG inhibition improves the prognosis of liver cancer through autophagy regulation and tumor immunity enhancement

Currently, the primary treatment for hepatocellular carcinoma (HCC) is a comprehensive treatment based on surgery.[1] In the case of advanced HCC that may not be removed surgically, additional issues, such as drug resistance and drug inefficacy with long-term use of chemotherapeutic drugs, highlight the pressing need for new treatment strategies.[2] Autophagy plays an essential role in cellular physiology, which was reported to modulate components of the immune system.[3] Autophagy-related immune genes (ARIGs) are linked to both autophagy and immunity. Here we identified histone deacetylase 1 (HDAC1), an ARIG strongly expressed in HCC, as a therapeutic target.[4] Then valproic acid (VPA), a specific inhibitor of HDAC1,[5] was used to treat liver cancer *in vitro* and *in vivo*. The results demonstrated that VPA could significantly induce autophagy and apoptosis of Hepa1-6 cells and inhibit tumor growth *in vivo*. This effect could be related to the regulation of autophagy and tumor immune microenvironment by VPA.

To identify differentially expressed genes in HCC, bioinformatics analysis was first performed. As illustrated in Figure 1A and Figure S1A, 23 ARIGs were differentially expressed in tumor tissues compared to normal liver tissues (log2 (Fold Change) = 1). The list of differentially expressed ARIGs is presented in Table 1. Moreover, enrichment analysis showed that the roles of these genes were associated with the apoptosis signaling pathway (Fig. S1B). Among the differentially expressed ARIGs, the vast majority of genes were highly expressed within tumor tissues (21 of 23; Fig. 1B), depicting that autophagy concerning immune processes occurs actively among tumor tissues.

Subsequently, the association between ARIGs and prognosis in HCC patients was investigated. After combining the survival data from HCC patients with gene expression data, Cox regression analysis revealed that 13 ARIGs were associated with patient prognosis, and all of the prognosis-related genes were high-risk genes (Hazard ratio >1; Fig. S1C). All patients were divided into two groups based on the median value of gene expression. The survival analysis showed that the patients in the low expression group of prognosis-related genes revealed a longer survival time (Fig. S1D). Following stepwise Cox regression analysis of the 13 ARIGs, four genes were utilized to construct the prognostic model, and the risk plots of the prognostic model are depicted in Figure 1C and Figure S2A. The survival time of patients within the low-risk group was significantly higher than that of the patients in the high-risk group. Moreover, the accuracy of this prediction score was significantly higher than that of single-factor predictors, including age and gender (Fig. S2B, C). Therefore, like many clinical indicators, the risk scoring system constructed using the four ARIGs can be used as an independent prognostic factor for patient prognosis (Fig. S2D).

Then, the expression of the four ARIGs was examined at the TCGA and cellular levels. ARIGs (BIRC5, HDAC1, PRKCD, SQSTM1) were significantly upregulated within liver cancer tissues than in normal liver tissues (Fig. S2E). Expression of these four ARIGs was elevated in murine hepatoma cell lines Hepa1-6 and H22 than in murine hepatocyte cell line AML12 (Fig. 1D). Likewise, the expression of the four ARIGs was increased in most human hepatoma cells than in the human hepatocyte line HL7702 (Fig. 1E). Since the four ARIGs are highly expressed within liver cancer, we chose to treat Hepa1-6 cells using hydroxychloroquine sulfate (HCQ, H141480, Aladdin), a classic autophagy inhibitor, to observe the impact of inhibiting autophagy on the four ARIGs. As illustrated by Figure S2F and S3A, HCQ could successfully inhibit the autophagy process of Hepa1-6 cells. Furthermore, we observed that HCQ could significantly upregulate the expression of acetylated histones within Hepa1-6 cells (Fig. S2G). In our previous results, HDAC1 was highly expressed in tumors, so it was speculated that there could

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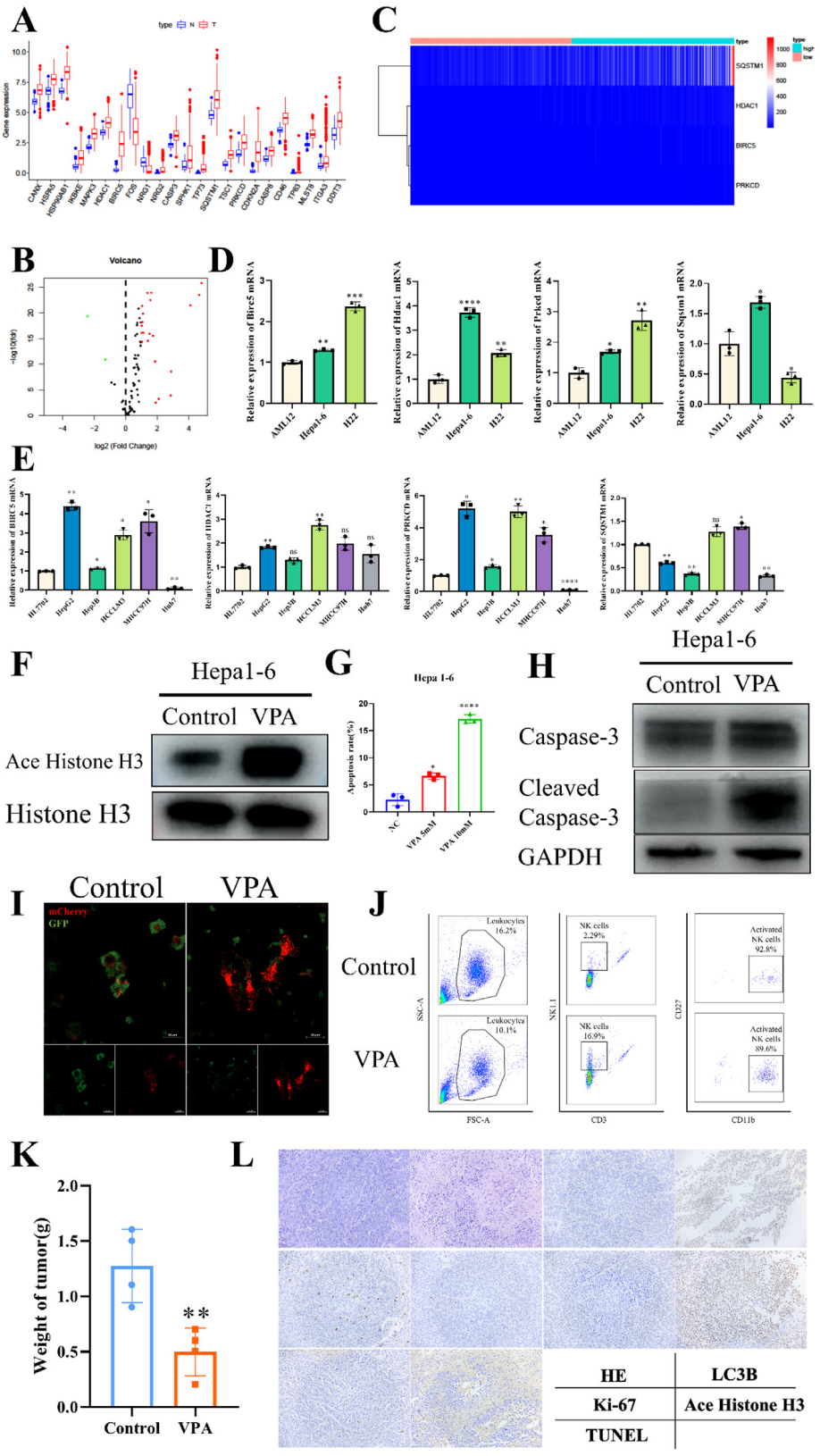

Figure 1 ARIG inhibition improves the prognosis of liver cancer through autophagy regulation and tumor immunity enhancement. **(A)** The boxplot of 23 differentially expressed ARIGs. The blue box represents normal liver tissue, and the red box represents tumor tissue. **(B)** The volcano map of 23 differentially expressed ARIGs. Red dots represent an upregulated expression, and green dots represent a down-regulated expression. **(C)** The risk plots of four ARIGs used to construct a prognostic risk model. **(D)** The RNA expression levels of four ARIGs used to construct prognostic risk models in murine hepatocellular carcinoma cell lines. *$P < 0.05$,

be some relationship between autophagic activity and histone acetylation.

HDAC inhibitors interfere with the function of histone acetylases by regulating the tightness of DNA wrapping around histones. We accidentally observed that inhibiting autophagy could induce the expression of acetylated histones. Therefore, VPA, a specific inhibitor of HDAC1, was chosen to treat Hepa1-6 cells and observe what happens after inhibiting HDAC1. Firstly, we observed the changes of HDAC1 in RNA levels among Hepa1-6 cells after VPA treatment. Surprisingly, the expression of HDAC1 at the RNA level was elevated upon VPA administration (Fig. S3B). It is possible that the inhibition of HDAC1 by VPA occurred after transcription, which caused a compensatory increase within HDAC1 transcription. Therefore, we detected the target protein of HDAC1. When VPA was treated, the acetylated histone expression was significantly increased, suggesting that VPA can functionally inhibit HDAC1 (Fig. 1F). Next, we tested the killing effect of VPA over HCC cells. The results indicated that VPA could significantly induce liver cancer cell apoptosis (Figs. 1G, H; Fig. S3C) and inhibit cancer cell proliferation (Fig. S3D). The killing effect increases with the administration concentration, reflecting a good tumor treatment effect. When HCC cells were treated using a moderate concentration of VPA, the intracellular autophagy process was significantly activated (Fig. 1I; Fig. S3E). This indicates that VPA-induced autophagy could be involved in cell apoptosis.

VPA acts as an inhibitor of HDAC1, and in our above results, HDAC1 belongs to one of the ARIGs. We will further explore whether VPA can improve the immune microenvironment in solid tumors. GSEA results show that immune responses are significantly enriched in high-risk groups (Fig. S4A). Compared with the high-risk group, the content of activated NK cells within the low-risk group was significantly enhanced (Fig. S4B), and the NK cell score was significantly increased (Fig. S4C). NK cells are immune cells in the body and activated NK cells can non-specifically kill the tumor cells. We analyzed the survival of patients in the high and low-risk groups, and the results revealed that the higher the content of activated NK cells, the longer the survival of patients (Fig. S4D). Simultaneously, the results of correlation analysis indicated that HDAC1 was negatively correlated with the content of activated NK cells (Fig. S4E), which indicated that we could inhibit HDAC1 from enhancing the activation of NK cells or increasing the content of NK cells, thereby killing tumors. Next, we constructed the Hepa1-6 tumor-bearing mice and performed flow cytometry analysis of NK cells in the tumor after two weeks of peritumoral administration of VPA. The results depict that VPA treatment can significantly increase the content of NK cells and activate NK cells inside the tumor (Fig. 1J; Fig. S4F).

A tumor-bearing mouse model was constructed on C57 mice with Hepa1-6 cell line and then treated with VPA. As shown in Figure S5A, after two weeks of administration, tumor growth was significantly inhibited, and tumor mass was significantly reduced in the VPA group compared with the control group (Fig. 1K), while the body weight of the mice did not change significantly (Fig. S5B). The immunohistochemistry results showed that after VPA treatment, large areas of necrosis and apoptosis appeared within the tumor tissue, and the proliferation ability of the tumor tissue was significantly weakened (Fig. 1L). Although the expression of HDAC1 in liver cancer tissues is significantly increased (Fig. S5C), the acetylation of HDAC1 target protein histones is significantly elevated upon VPA treatment and this process is accompanied by autophagy (Fig. 1L).

Ethics declaration

This study was approved by the Stomatology Hospital, School of Stomatology, Zhejiang University School of Medicine, Zhejiang, China.

Author contributions

Y.Z. and Y.B. conceived and designed the study. J.Q.C. and X.T.Z. conducted the study. W.J.H. contributed to the acquisition of data. Y.B. and J.Q.C. analyzed and interpreted the data. Y.Z. and Y.B. reviewed and edited the article. All authors read and approved the final version of the manuscript.

Conflict of interests

The authors declared that they have no competing interests.

Consent for publication

All authors agreed to publish this manuscript.

Data availability

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gendis.2022.10.007.

$^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$, versus normal murine hepatocyte line. **(E)** The RNA expression levels of four ARIGs used to construct prognostic risk models in human hepatocellular carcinoma cell lines. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{****}P < 0.0001$, versus the normal human hepatocyte line. **(F)** The expression of acetylated histone in Hepa1-6 cells before and after VPA treatment. **(G)** The quantitative results of Hepa1-6 cells before and after VPA treatment. $^{*}P < 0.05$, $^{****}P < 0.0001$, versus the control group. **(H)** The expression of apoptosis-related proteins in Hepa1-6 cells before and after VPA treatment. **(I)** The changes in autophagic flux in Hepa1-6 cells before and after VPA treatment. **(J)** The flow cytometric analysis of intratumoral NK cells in tumor-bearing mice. **(K)** The changes in tumor weight before and after VPA treatment. $^{**}P < 0.01$, versus the control group. **(L)** The immunohistochemical results of tumors in tumor-bearing mice before and after VPA treatment.

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