Dear editor:

Our recent research has found that the kynurenine derivative 3-HAA was lower in tumour cells due to the downregulation of its synthetic enzyme kynurenine 3-monooxygenase (KMO), and overexpression of KMO suppressed hepatocellular carcinoma (HCC) tumour formation and tumour growth by increasing endogenous 3-HAA. It is well known that kynurenine promotes tumour growth by directly binding to the aryl hydrocarbon receptor.1,2,3 The 3-hydroxyanthranilic acid (3-HAA), a derivative of kynurenine, was reported to induce apoptosis by upregulating phosphatases.4 However, the metabolism and function of kynurenine derivatives largely remain unclear. Here, we report our novel findings related to kynurenine metabolism.

3-HAA is decreased in tumour cells. Tryptophan catabolites were first analysed in clinical HCCs. The concentration of kynurenine catabolite 3-HAA decreased in both HCC and oesophageal carcinomas compared to the matched paratumour tissues (Figure 1A; Figure S1A).
Conversely, the concentration of tryptophan and kynurenine was higher in these HCCs and oesophageal carcinomas than in the matched paratumour tissues, respectively. Consistent with this observation, the concentration of 3-HAA was also lower in seven HCC cell lines tested than in normal hepatic cells, whereas the content of tryptophan and kynurenine increased in these tested HCC cell lines (Figure 1B). The immunohistochemistry analysis further confirmed lower 3-HAA content in clinical HCC tissues than in adjacent non-cancerous tissues (Figure 1C).

Metabolic flux analysis revealed tryptophan metabolised to kynurenine but not 3-hydroxykynurenine (3-HK) or 3-
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FIGURE 3 KMO overexpression inhibits tumour formation by inducing apoptosis. (A) The effect of HAAO and KMO on the cell growth of SMMC7721 cells. The KMO and HAAO were overexpressed or knocked down in SMMC7721 cells, separately. *: p < .05, **: p < .01. (B) The effect of KMO overexpression on apoptosis of SMMC7721 cells. *: p < .05. (C) The effects of three types of inhibitors on 3-HAA-induced HCC cell death. ZVAD: apoptosis inhibitor (20 μM); Nec1: necrosis inhibitor (100 μM); 3-MA: autophagy inhibitor (5 mM). The dose of 3-HAA was...
HAA in HCC cells, and the newly generated kynurenine was secreted into the culture medium (Figure 1D), suggesting 3-HAA is decreased in tumours, at least in HCCs and oesophageal carcinomas.

**Upregulation of KMO increases 3-HAA.** To determine whether the metabolic enzymes regulate 3-HAA concentration, we assessed the expression of 3-HAA-related enzymes in HCC cells. The immunoblotting and immunohistochemistry analysis showed that KMO and kynureninase (KYNU) were downregulated in HCC cells and tissues. In contrast, the indoleamine 2,3-dioxygenase 1 (IDO1) and tryptophan 2,3-dioxygenase (TDO2) was upregulated (Figure 2A, B). This finding was consistent with the HCC expression profile in the TCGA database (Figure 2C). Moreover, both KMO and KYNU expression (www.gtexportal.org) are commonly downregulated in tumours originated from tissues abundantly expressing KMO and KYNU. These tumours include lung, kidney, and liver carcinomas, which are the top 10 tumours worldwide in terms of death (Figure 2D).

In addition, overexpression of KMO significantly increased the concentration of 3-HAA in HCC SMMC7721 cells, but not the 3-HK, picolinate (PA), or quinolinate (QA; Figure 2E). The hydroxyanthranilate-3,4-dioxygenase (HAAO) knockdown had similar effects on the levels of these metabolites (Figure 2F).

**KMO overexpression inhibits tumour formation by inducing apoptosis.** Functionally, either overexpression of KMO or knockdown of HAAO inhibited cell growth of HCC cells in vitro by increasing apoptosis (Figure 3A, B). Only the apoptosis inhibitor zVAD restored growth of HCC cells following 3-HAA treatment or overexpressing KMO (Figure 3C, D). Moreover, KMO overexpression suppressed tumour formation and tumour growth in the HCC xenograft nude mice model (Figure 3E; Figure S2A).Remarkably, the Kaplan–Meier survival analysis showed that HCC patients with high KMO expression had a prolonged disease-free survival than patients with low KMO expression (Figure 3F). The 3-HAA treatment significantly inhibited HCC cell growth and colony formation (Figure 3G; Figure S2B). Moreover, 3-HAA but not kynurenine slowed tumour growth in a CDX model and in a patient-derived xenograft (PDX) model (Figures S2C and 3H), suggesting KMO overexpression inhibits tumour formation and tumour growth via its catabolite 3-HAA.

Through gene expression profiling, real-time PCR and immunoblotting, the top two upregulated genes DUSP6 and IGFBP1 were selected for further study (Figure 3I, J). However, the clinical data showed that the overall survival of HCC patients was only associated with the expression level of DUSP6, but not IGFBP1 (Figure 3K; Figure S2D). Patients expressing a high level of DUSP6 showed a more prolonged overall survival than patients expressing a low level of DUSP6 (Figure 3K), and the corrective analysis with the clinical characteristics also supported this finding (Figure S2E). Also, we demonstrated that DUSP6 mediates 3-HAA-induced tumour cell apoptosis via ERK signalling (Figure 3L, M; Figure S2F, G), which was consistent with our previous finding.

According to the fact that 3-HAA activates transcription factor YY1,4,5 closer analysis of the DUSP6 promoter region using online-based prediction tools6,7 revealed a novel potential YY1 binding DNA fragment at positions −1145 to −1134, which was distinct from the reported consensuses binding sequence.8 This finding was further confirmed by a luciferase assay and ChIP-QPCR (Figure S2H, I). The TUNEL assay demonstrated that 3-HAA-induced apoptosis was reduced in SMMC7721 cells depleted of YY1, overexpression of DUSP6 restored the apoptosis suppressed by YY1 depletion (Figure S2J).

**KMO enhances the inhibition effect of IDO1 inhibitor on HCC growth.** The various HCC mouse models were implemented to further evaluate the potential application of KMO target in clinics. As shown in Figure 4A, DUSP6 knockdown reversed KMO-mediated suppression of tumour growth in SMMC7721 xenografts.
KMO enhances the inhibition effect of IDO1 inhibitor on HCC growth. (A) DUSP6 knockdown recovered KMO-suppressed xenograft growth. SMMC7721 cells overexpressing KMO were subcutaneously injected into mice. The middle graph shows xenograft weights in different groups. Photographs on the right show representative tumours in different groups (n = 6). **: p < .01. (B) DUSP6 knockdown restored KMO-reduced tumour numbers and shortened the survival of mice bearing transposon HCCs. The genetic transposon HCC mouse model was established as described in the section of methods and materials (n = 6). (C) KMO overexpression enhances the inhibition effect of IDO1 inhibitor Epacadostat to suppress HCC xenograft growth. The mouse liver cancer Hep1-6 cells (1 × 10⁶) were inoculated into immune-competent C57BL/6 mice (n = 6). *: p < .05. (D) KMO overexpression prolongs the survival of mice bearing transposon HCCs with IDO1 inhibitor Epacadostat. The dose of Epacadostat (IDO1 inhibitor) was 100 mg/kg/day. Note: The mouse xenografts were generated by the inoculation of 1.5 × 10⁶ of SMMC7721 cells into the armpit of the rear limb. Tumour volumes are presented as mean ± SD (*: p < .05; **: p < .01). Photographs show representative xenografts in different groups. (E) The working model.

More impressively, KMO overexpression reduced the tumour numbers and prolonged the survival in a transposon HCC mouse model. DUSP6 depletion promoted tumour formation and shorten mice survival, and KMO overexpression had little effect on tumour formation and mice survival after DUSP6 knockdown (Figure 4B).

Most importantly, KMO enhances the effect of IDO1 inhibitor Epacadostat to suppress HCC xenograft growth in an immune-competent mouse model (Figure 4C). In the meantime, the combination of KMO overexpression with IDO1 inhibitor Epacadostat also inhibited the HCC tumour growth and prolonged the
survival of mice bearing transposon-induced HCCs (Figure 4D).

In brief, this study reveals that both KMO and its substrate 3-HAA decreases in HCC cells and HCC tissues. The KMO overexpression as well as 3-HAA treatment reverses the tumour-promoting effect of kynurenine and significantly improves the efficacy of IDO1/2 inhibitors on HCC xenografts (Figure 4E). These findings show that downregulation of KMO appears to be essential for HCC growth, suggesting the kynurenine metabolic enzyme KMO is a promising therapeutic target for HCC.

CONSENT FOR PUBLICATION
Not applicable.

CONFLICT OF INTEREST
The authors declare that they have no competing interests.

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REFERENCES
1. Opitz CA, Litzenburger UM, Sahm F, et al. An endogenous tumour-promoting ligand of the human aryl hydrocarbon receptor. *Nature*. 2011;478:197-203.
2. Li Y, Innocentin S, Withers DR, et al. Exogenous stimuli maintain intraepithelial lymphocytes via aryl hydrocarbon receptor activation. *Cell*. 2011;147:629-640.
3. Schwarcz R, Bruno JP, Muchowski PJ, Wu HQ. Kynurenines in the mammalian brain: when physiology meets pathology. *Nat Rev Neurosci*. 2012;13:465-477.
4. Gan G, Shi Z, Shangguan C, et al. The kynurenine derivative 3-HAA sensitizes hepatocellular carcinoma to sorafenib by upregulating phosphatases. *Theranostics*. 2021;11:6006-6018.
5. Shi Z, Gan G, Xu X, et al. Kynurenine derivative 3-HAA is an agonist ligand for transcription factor YY1. *J Hematol Oncol*. 2021;14:153.
6. Khan A, Fornes O, Stigliani A, et al. JASPAR 2018: update of the open-access database of transcription factor binding profiles and its web framework. *Nucleic Acids Res*. 2018;46:D1284.
7. Khan A, Fornes O, Stigliani A, et al. JASPAR 2018: update of the open-access database of transcription factor binding profiles and its web framework. *Nucleic Acids Res*. 2018;46:D260-D266.
8. Kim J, Kim J. YY1's longer DNA-binding motifs. *Genomics*. 2009;93:152-158.

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