Research Article

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Monoamine oxidase A suppresses hepatocellular carcinoma metastasis by inhibiting the adrenergic system and its transactivation of EGFR signaling

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Background & Aims: Monoamine oxidase A (MAOA), a catecholamine neurotransmitter degrading enzyme, is closely associated with neurological and psychiatric disorders. However, its role in cancer progression remains unknown.

Methods: Hepatocellular carcinoma (HCC) tissue arrays (n = 254) were used to investigate the correlation between MAOA expression and clinicopathological findings. *In vitro* invasion and anoikis assays, and *in vivo* intrahepatic and lung metastasis models were used to determine the role of MAOA in HCC metastasis. Quantitative real-time PCR, western blotting, immunohistochemical staining and HPLC analysis were performed to uncover the mechanism of MAOA in HCC.

Results: We found that MAOA expression was significantly downregulated in 254 clinical HCC samples and was closely correlated with cancer vasoinvasion, metastasis, and poor prognoses. We then demonstrated that MAOA suppressed norepinephrine/epinephrine (NE/E)-induced HCC invasion and anoikis inhibition, and uncovered that the effects of NE/E on HCC behaviors were primarily mediated through alpha 1A (ADRA1A) and beta 2 adrenergic receptors (ADRB2). In addition to the canonical signaling pathway, which is mediated via adrenergic receptors (ADRs), we found that ADR-mediated EGFR transactivation was also involved in NE-induced HCC invasion and anoikis inhibition. Notably, we found that MAOA could synergize with EGFR inhibitors or ADR antagonists to abrogate NE-induced HCC behaviors.

Conclusions: Taken together, the results of our study may provide insights into the application of MAOA as a novel predictor of clinical outcomes and indicate that increasing MAOA expression or enzyme activity may be a new approach that can be used for HCC treatment.

Keywords: Monoamine oxidase A; Hepatocellular carcinoma; Metastasis; Adrenergic receptor; EGFR.

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Abbreviations: ADAM, a disintegrin and metalloproteinases; ADRA1A, α1A adrenergic receptors; ADRB2, β2 adrenergic receptors; ADR, adrenergic receptor; cDNA, complementary DNA; CNL, corresponding noncancerous liver; DFS, disease-free survival; DMEM, Dulbecco's modified Eagle's medium; E, epinephrine; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; Erk, extracellular signal-regulated kinase; FBS, fetal bovine serum; GPCRs, G protein-coupled receptors; HB-EGF, heparin-binding EGF; HCC, hepatocellular carcinoma; H&E, hematoxylin and eosin; HPLC, high performance liquid chromatography; MAOA, monoamine oxidase A; MAPK, mitogen-activated protein kinase; MMP, metalloproteinases; mRNA, messenger RNA; NE, norepinephrine; NS, no significance; OS, overall survival; PI, propidium iodide; siRNA, small interfering RNA; TNM, tumor-node-metastasis.

Introduction

Cancer metastasis may be affected by many factors, including intracellular signaling molecules and extracellular components, such as cytokines; the extracellular matrix; and neurotransmitters [1,2]. Growing evidence indicates that the nervous system plays important roles in cancer progression [3]. Recently, it has been reported that sympathetic and parasympathetic nervous systems contribute to cancer development and dissemination in prostate cancer [4]. Adrenergic neurotransmitters, norepinephrine (NE) and epinephrine (E), have been reported to promote cancer cell migration and invasion in multiple types of cancer via β-ARs [5,6]. Furthermore, besides cancer cells, stromal cells in the tumor microenvironment could also be affected by the nervous system [7,8]. Studies of ovarian cancer have demonstrated that NE/E may contribute to tumor progression by promoting angiogenesis [9]. By a large-scale cDNA transfection screening, our previous study revealed that many neurotransmitter receptor-related genes are closely associated with cancer cell proliferation and survival [10]. We further found that acetylcholinesterase acts as a tumor growth suppressor in regulating cell proliferation,

the relevant signaling pathway and the drug sensitivity of HCC cells [11].

The major NE/E degrading enzyme is monoamine oxidase A (MAOA), which catalyzes the oxidative deamination of monoamines. In addition to neurons and astrocytes, MAOA is also expressed in the liver, digestive tract, and placenta [12,13]. MAOA is closely associated with human emotional and mental activity. Several reports have demonstrated that reduced MAOA activity is related to human social and criminal behaviors [14–16]. Few studies have addressed the role of MAOA in cancer except for prostate cancer and cholangiocarcinoma, whereas diverse roles were displayed [17–19]. By analyzing three independent HCC microarray datasets from the GEO database, we found that *MAOA* mRNA levels are significantly downregulated in the majority of HCC tissues compared to non-tumorous liver tissues.

In the present study, we found that *MAOA* was significantly downregulated in HCC by epigenetic alteration and the expression of *MAOA* was closely associated with the key events of tumor metastasis. Further investigations have demonstrated that in addition to the canonical signaling pathway mediated via ADRs, NE-induced HCC invasion and anoikis inhibition are partially mediated by EGFR transactivation. Therefore, EGFR inhibitors, antagonists of ADRs or MAOA overexpression can synergistically inhibit NE-induced HCC metastatic behaviors, thereby indicating that MAOA may be a metastatic and prognostic predictor. In addition, increasing MAOA expression or enzyme activity may be a novel approach that can be used for HCC therapy.

Materials and methods

Materials and methods of cell culture, clinical samples, *in vitro* assays, *in vivo* studies, and statistical analysis are provided in Supplementary Materials and methods.

Cancer

Results

MAOA is downregulated in HCC and is closely related to blood vessel invasion and patient prognoses

By analyzing three independent HCC microarray datasets from the GEO database, we found that the mRNA levels of *MAOA* were significantly downregulated in the majority of HCC tissues compared to non-tumorous tissues (Table 1). In the present study, we examined the expression levels of MAOA in 254 HCC and corresponding non-cancerous liver (CNL) tissues using immunohistochemical staining (Fig. 1A). The results indicated that MAOA was downregulated in 70.5% (179/254) of HCC patients (Fig. 1B). In HCC cell lines, MAOA expression levels were lower in most tested HCC cells, with the exceptions of SK-Hep1 and SNU-398, compared to immortalized human liver L-O2 cells (Supplementary Fig. 1A and B).

To investigate the clinical significance of MAOA in HCC, we analyzed the MAOA expression status with respect to various pathological parameters in 254 HCC patients. The results indicated that MAOA expression in HCC tissues were closely correlated with blood vessel invasion, tumor thrombus formation, tumor encapsulation, TNM stage, tumor size, alpha-fetoprotein levels, and tumor differentiation (Table 2). Furthermore, high MAOA expression was associated with improved overall survival (OS) (Fig. 1C) and disease-free survival (DFS) (Fig. 1D). Taken together, our data strongly implied that MAOA may act as an indicator of HCC metastasis and prognosis. To further investigate the relevance of MAOA with HCC metastasis, we collected normal liver, CNL, HCC, and tumor thrombus tissues from the same patients and characterized MAOA expression in these tissues. As demonstrated in Fig. 1E, MAOA expression was higher in the normal liver tissues and CNL, and lower in the HCC and tumor thrombus samples. These results were further confirmed by western blotting (Fig. 1F). Using 5-aza-2′-deoxycytidine (DAC), a specific methyltransferase inhibitor, and Trichostatin A (TSA), a histone deacetylase inhibitor, we found that methylation occurred in all of 5 tested HCC cell lines, and histone acetylation existed in 3 of them. These data indicated that epigenetic methylation and histone acetylation suppress the expression level of MAOA in HCC (Supplementary Fig. 2).

MAOA suppresses NE/E-induced HCC cell invasion and anoikis inhibition in vitro and HCC metastasis in vivo

Previous studies have demonstrated noticeable effects of NE/E on cancer cell invasion and anoikis [6,7], but the role of the adrenergic system in HCC cell behavior has not been reported. In our study, we found that NE/E promoted HCC invasion and prevented anoikis in both SMMC-7721 and MHCC-97L cells (Fig. 2A and B). And these effects were completely abrogated by MAOA overexpression (Fig. 2A and B and Supplementary Fig. 1C). However, NE/E had no effect on HCC cell proliferation (Supplementary Fig. 3).

To further explore the role of MAOA in cancer progression *in vivo*, Lenti-vector or Lenti-*MAOA* cells (SMMC-7721) were orthotopically injected into nude mice to assess intrahepatic metastases, and intravenously transplanted to assess distant metastases. Histological examinations of the liver and lung tissues indicated that mice transplanted with MAOA overexpressing cells had fewer intrahepatic and pulmonary metastatic nodules than those transplanted with control cells (Fig. 2C and D).

To further confirm that MAOA is a metastasis suppressor *in vivo*, we used a specific MAOA inhibitor, clorgyline, to inhibit endogenous MAOA activity in HCC cells in nude mice. SK-Hep1, an HCC cell line with high endogenous MAOA, was orthotopically or intravenously transplanted into nude mice. Clorgyline (0.2 mg) was then intraperitoneally injected into nude mice twice per week and the animals were sacrificed 6 weeks later. The orthotopically transplanted mice treated with clorgyline had greater numbers of intrahepatic metastatic nodules than those treated with the vehicle (Fig. 2E). No pulmonary metastatic nodules were observed in the intravenous transplantation models.

MAOA reduces NE/E levels in vitro and MAOA expression is inversely correlated with NE/E levels in HCC tissues

To explore the mechanism underlying MAOA suppresses NE/E-induced HCC cell behavior, we tested whether MAOA can remove NE/E from the HCC microenvironment. After 6 h incubation, the concentrations of exogenous NE/E were determined to be approximately 30% less in MAOA overexpressing cells than in control cells (Supplementary Fig. 4).

Further, NE/E levels in HCC tissues were found to be inversely correlated with MAOA expression (Fig. 3A and B). Interestingly, our data also indicated that the predominant catecholamine neu-

Table 1. Analysis of MAOA expression in 3 independent HCC microarray data from GEO database.

GSE serials	HCC/CNL	*MAOA* expression		
		Cases	Down	Up
GSE6764	Early or advanced HCC compared with normal liver tissue	35	33 (94%)	2 (6%)
GSE11260	HCC compared with adjacent non-tumor tissue	32	26 (81%)	6 (19%)
GSE14323	Liver tissue with HCC compared with normal liver tissue	55	55 (100%)	0 (0%)

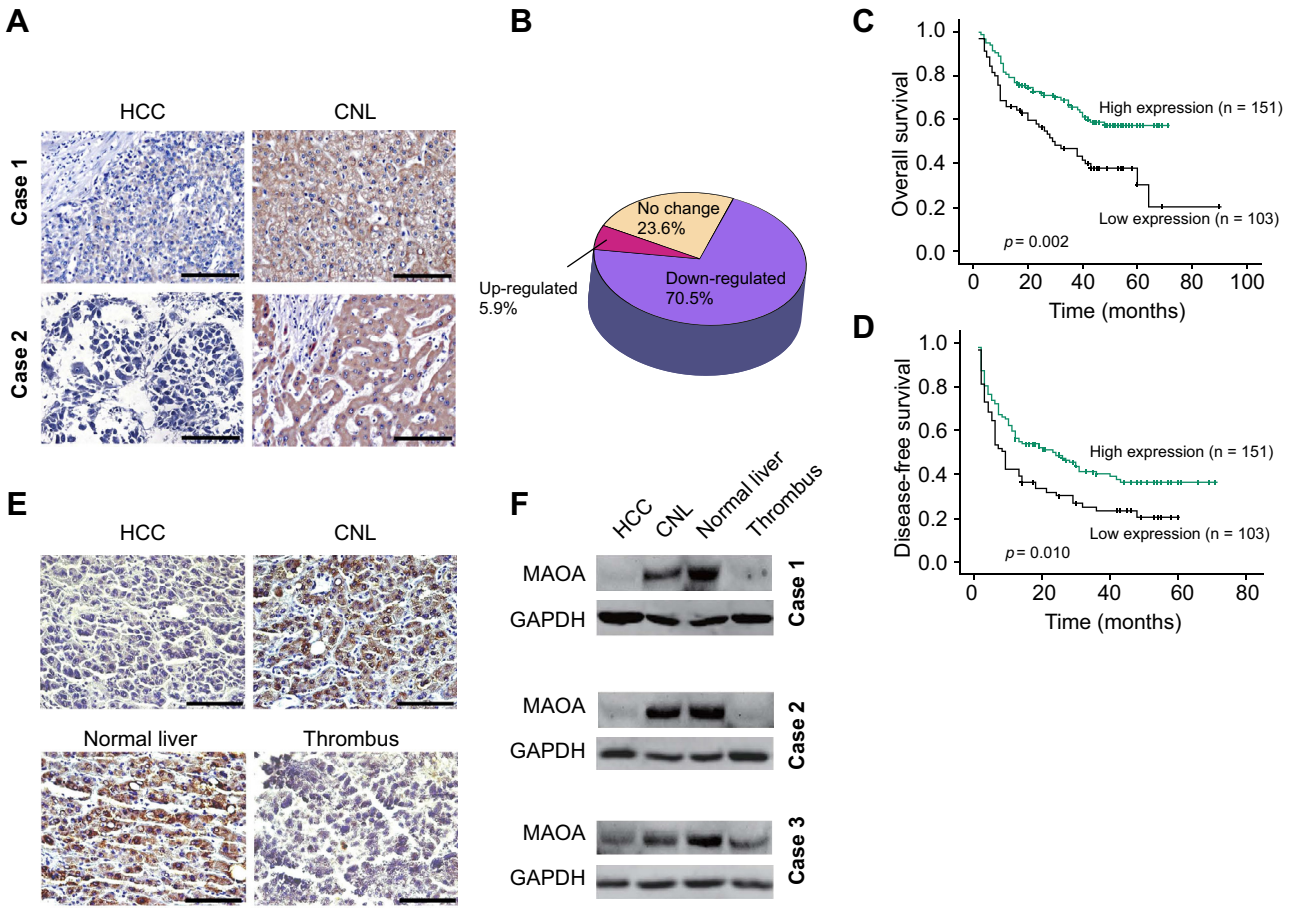


Fig. 1. MAOA is down-regulated and is closely related to patient prognosis in hepatocellular carcinoma (HCC). (A) The immunohistochemical staining of MAOA in HCC and corresponding noncancerous liver (CNL) tissues. Scale bars, 10 μm. (B) The expression of MAOA was down-regulated in 70.5% of HCC patients. (C) Kaplan-Meier analysis of OS for the expression of MAOA. $p = 0.002$. (D) Kaplan-Meier analysis of DFS for the expression of MAOA. $p = 0.010$. (E) The immunohistochemical staining of MAOA in HCC, CNL, normal liver and thrombus tissues. Scale bars, 10 μm. (F) Western blotting analysis of MAOA expression in HCC, CNL, normal liver and thrombus tissues. GAPDH was used as a loading control.

rotransmitter in HCC tissues was NE, which was 98.9 times higher than the levels of E (Fig. 3C). Dopamine and serotonin were undetectable in HCC tissues. Additionally, MAOA was also detected in the cell culture supernatants of overexpressed SMMC-7721 or MHCC-97L cells, suggesting that MAOA can degrade extracellular NE/E in the HCC microenvironment (Fig. 3D).

NE/E promote HCC cell invasion via α1A- and β2-ADRs and prevent anoikis via the β2-ADR

To identify the ADR subtypes that mediate NE/E-induced HCC cell invasion and anoikis inhibition, we examined the expression patterns of ADRs by RT-PCR and western blot analysis. We found that ADRA1A and ADRB2 are the predominate subtypes of 9 ADRs in HCC tissues and cell lines (Supplementary Fig. 5A–D), which is consistent with previous reports indicating that the ADRA1A- and ADRB2-subtypes are the predominant ADRs that are expressed in the human liver plasma membrane [20].

We further explored the functional roles of ADRA1A and ADRB2 in NE/E-induced HCC cell invasion and anoikis inhibition. Prazosin (an α1-ADR antagonist, 100 nmol/L) and ICI118551 (a β2-ADR antagonist, 100 nmol/L) were applied. ICI118551 can effectively inhibit NE/E-induced SMMC-7721 cell invasion and anoikis inhibition, while prazosin can only inhibit NE/E-induced SMMC-7721 cell invasion (Fig. 3E and Supplementary Fig. 6A). Similar effects were observed using small interfering RNA (siRNA) to downregulate the expression of ADRA1A and ADRB2 (Supple-

Table 2. Correlation of the clinicopathological findings with MAOA expression.

Variable	MAOA (n)		
	High	Low	*p* value
Age			
≤50 yr	65	55	0.105
>50 yr	86	48	
Gender			
Female	20	14	0.936
Male	131	89	
Hepatitis history			
Yes	117	70	0.752
No	10	5	
Gamma-glutamytransferase			
≤50 (U/L)	42	20	0.340
>50 (U/L)	85	55	
Alpha-fetoprotein			
≤20 ng/ml	52	15	0.002
>20 ng/ml	75	60	
Glu			
≤7 mmol/L	110	68	0.390
>7 mmol/L	17	7	
Lobe			
Right	81	51	0.795
Left	29	16	
Right & Left	17	8	
Tumor multiplicity			
Single	109	60	0.279
Multiple	18	15	
Tumor satellite			
Yes	17	16	0.140
No	110	59	
*Tumor encapsulation			
Incomplete	62	50	0.014
Complete	65	25	
**Tumor thrombus			
Yes	16	28	<0.001
No	111	47	
Tumor differentiation			
I	6	1	0.003
II	74	32	
III	71	70	
**Vascular invasion			
Yes	36	64	<0.001
No	115	39	
Tumor size			
≤5 cm	98	30	<0.001
>5 cm	53	73	
TNM stage			
I	105	49	<0.001
II	22	7	
III	24	47	

Pearson's χ^2 test was used.

mentary Fig. 5E). ADRB2 siRNA effectively inhibited NE/E-induced SMMC-7721 cell invasion and anoikis inhibition, while ADRA1A siRNA only inhibited NE/E-induced SMMC-7721 cell invasion (Fig. 3G and Supplementary Fig. 6C).

Another HCC cell line, MHCC-97L, in which only the β subtype of ADRs are expressed was used to determine predominant functional ADR subtypes (Supplementary Fig. 5C). The β2-AR antagonist ICI118551 effectively inhibited NE/E-induced MHCC-97L cell invasion and anoikis inhibition, while the α1-AR antagonist, prazosin, has no significant effects on NE/E-induced cell invasion or anoikis inhibition (Fig. 3F and Supplementary Fig. 6B). Similar effects were observed using siRNA interference (Fig. 3H and Supplementary Figs. 5E and 6D).

These results indicated that NE/E-induced HCC cell invasion is mediated by both α1- and β2-ADRs, but anoikis inhibition is only mediated by β2-ADRs.

MAOA suppresses the ADR-mediated transactivation of EGFR signaling

Recent studies have shown that G protein-coupled receptors (GPCRs), including ADRs, transactivate EGFR signaling to modulate biological functions under specific physiological or pathological conditions [21]. Activation of GPCRs results in metalloprotease-dependent release of EGF-like ligands (HB-EGF, amphiregulin, TGF-alpha, etc.), which in turn activate EGFR and intracellular downstream signal [22]. NE/ADR-mediated EGFR transactivation was observed in several HCC cell lines and was more significant in cells (SMMC-7721, MHCC-97L, and Hep3B) expressing lower levels of MAOA than in cells (SK-Hep1) expressing relatively higher levels of MAOA (Fig. 4A).

SMMC-7721 cells, which have higher levels of EGFR transactivation following NE stimulation were used to further investigate the effects of MAOA on ADR-mediated EGFR transactivation (Fig. 4B). The results indicated that both NE and EGF can induce EGFR activation in SMMC-7721 cells, while MAOA overexpression significantly suppressed NE-, but not EGF-, induced EGFR phosphorylation and the downstream activation of MAPK/extracellular signal-regulated kinase (Erk1/2) (Fig. 4B and Supplementary Fig. 7).

Prazosin and ICI118551 were used to determine whether NE-induced EGFR transactivation is mediated via α1A- or β2-ADRs in SMMC-7721 cells. We found that both prazosin and ICI118551 reduced NE-induced EGFR phosphorylation. Similar inhibitory effects were also observed by using EGFR inhibitors AG1478 (100 nmol/L) and erlotinib (100 nmol/L). Additionally, protein kinase A (PKA), a canonical effector of NE-ADR signaling, was shown to be activated by NE stimulation (which was suppressed by treatment with ADR antagonists, but not EGFR inhibitors) (Fig. 4C). MAOA overexpression can suppress both NE-induced PKA activation and EGFR transactivation (Fig. 4C).

MMP7, ADAM12, and HB-EGF are involved in NE-induced EGFR transactivation

GPCR-mediated EGFR transactivation requires either specific metalloproteinases (MMPs) or disintegrin and metalloproteinases (ADAMs) to cleave EGF-like ligands [21,23]. To identify which MMPs, ADAMs or EGF-like ligands were involved in NE-induced transactivation of EGFR, we performed genome-wide cDNA microarrays with vehicle-treated Lenti-vector/SMMC-7721 cells, NE-treated Lenti-vector/SMMC-7721 cells and NE-treated Lenti-*MAOA*/SMMC-7721 cells. The results showed that *MMP7*, *ADAM12*, and *HB-EGF* expression were upregulated by

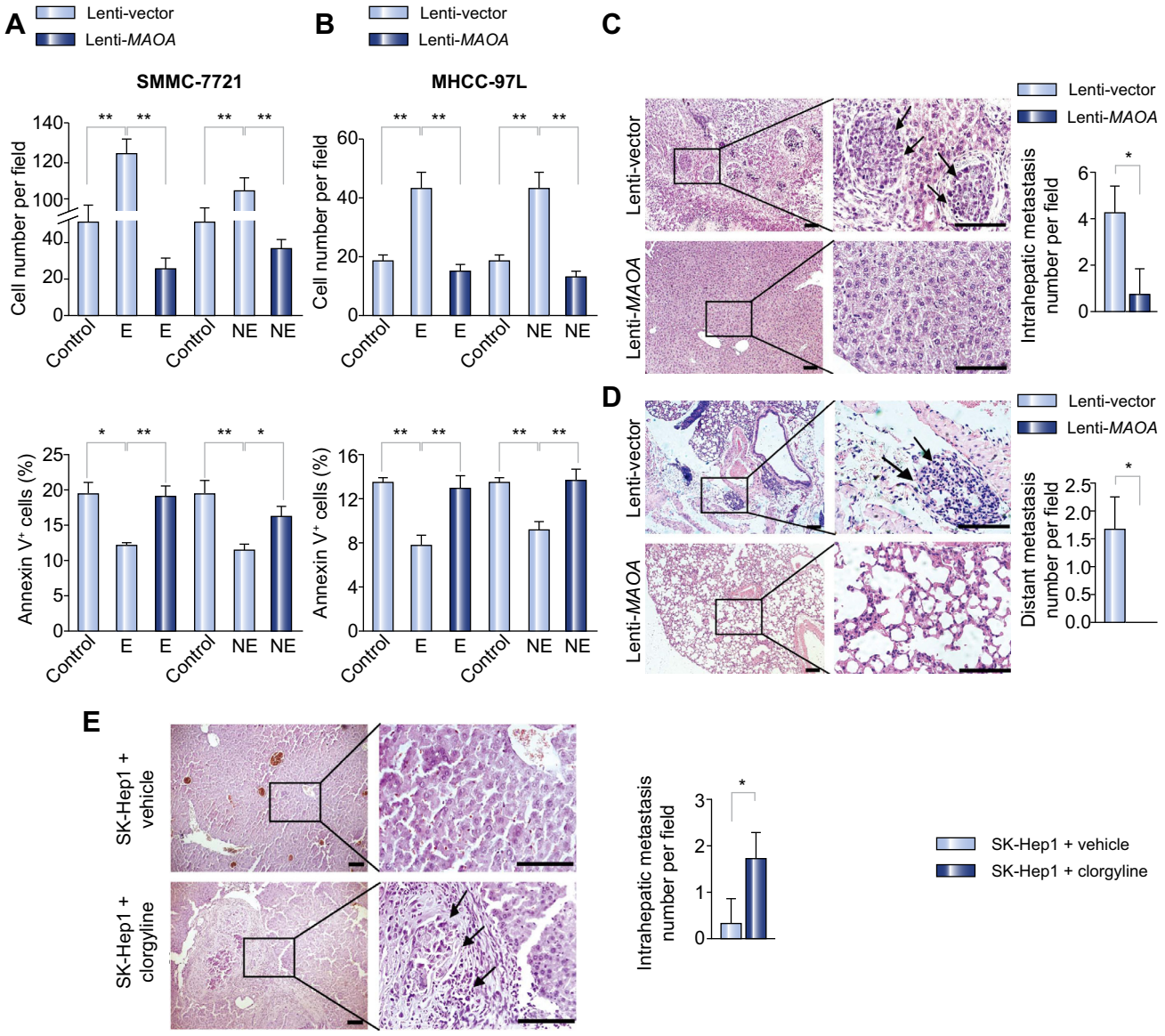


Fig. 2. MAOA overexpression abrogates the effects of epinephrine (E) or norepinephrine (NE) on HCC cell invasion and anoikis inhibition *in vitro* and metastasis *in vivo*. (A and B, upper) Representative quantification of cell invasiveness analysis of SMMC-7721 (A) and MHCC-97L (B) cells infected with Lenti-*MAOA* and Lenti-vector, stimulated by E or NE. (A and B, lower) Flow cytometry statistical analysis of anoikis of SMMC-7721 (A) and MHCC-97L (B) cells infected with Lenti-*MAOA* and Lenti-vector, stimulated by E or NE. (C) Representative images of H&E staining in liver tissues from mice orthotopically inoculated with MAOA overexpressed SMMC-7721 and control cells. Statistical analysis of intrahepatic metastatic focis (200×) in the two groups is shown right. (D) Pulmonary metastases were detected by H&E staining. Statistical analysis of numbers of pulmonary metastatic nodules (200×) is shown right. (E) H&E staining of intrahepatic tumors and metastases in mice orthotopically inoculated with SK-Hep1 cells and intraperitoneally injected with clorgyline or vehicle. Statistical analysis of intrahepatic metastatic focis (200×) in the two groups is shown right. Arrows: intrahepatic or pulmonary metastases. Scale bars, 10 μm $^{*}p$ <0.05, $^{**}p$ <0.01.

NE stimulation and this upregulation was abrogated by MAOA overexpression. But the expression of *MMP2*, *MMP9*, *ADAM10*, ADAM17, amphiregulin, and TGF-alpha were not altered by NE stimulation or MAOA overexpression (Table 3). These results were further confirmed with real-time PCR analyses (Fig. 4D). In addition, the activity of ADAM17, the main metalloprotease responsible for the shedding of the EGFR ligands, was unchanged under NE stimulation by measuring the activity of TACE/ADAM17 with fluorogenic substrate (Supplementary Fig. 8).

To further confirm that MMP7 and ADAM12 are the major proteinases involved in NE-induced transactivation of EGFR, we use RNA interference to knock-down their expression and specific inhibitors to block their activities. The results showed that NE-induced phosphorylation of EGFR was obviously suppressed by silencing of *MMP7* or *ADAM12* (Fig. 4E). The effects of NE on HCC cell invasion and anoikis were also abrogated by silencing of *MMP7* or *ADAM12* (Supplementary Fig. 9B and C). Additionally, similar results were obtained by application of MMP inhibitor III (a MMP7 inhibitor, 10 μmol/L) and GM6001 (a MMP7/ADAM12 inhibitor, 25 μmol/L) (Fig. 4F and Supplementary Fig. 9D and E).

To confirm that HB-EGF is the major EGF-like ligand involved in NE-induced EGFR transactivation in HCC cells, we further

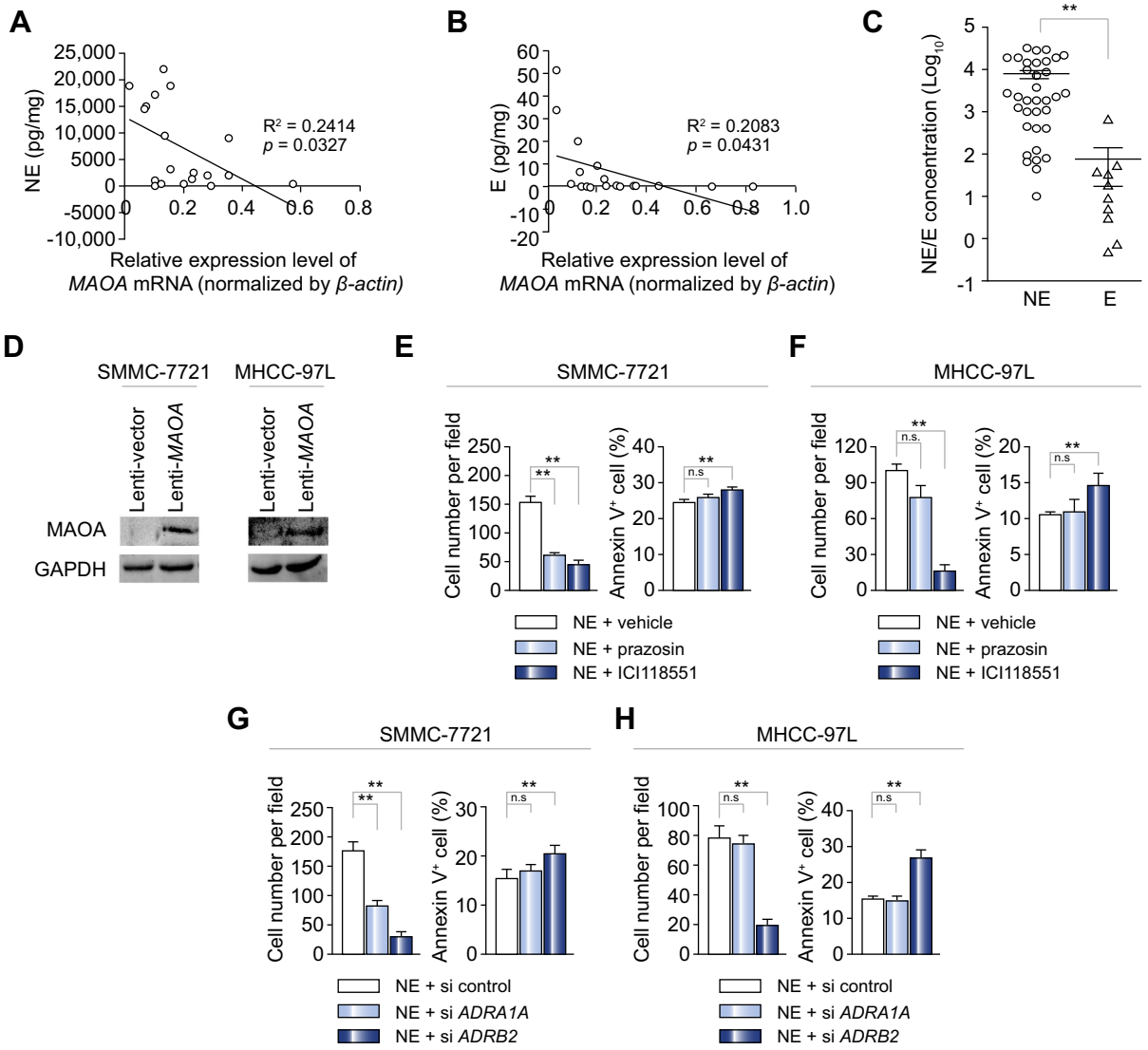


Fig. 3. The expression of MAOA is inversely correlated with NE/E content in HCC tissues, and NE-induced HCC cell invasion and anoikis inhibition were mediated by different adrenoceptors. (A) Analysis of correlation between the mRNA level of *MAOA* and the content of NE in 19 cases of human HCC tissues. Pearson's correlation was used. (B) Analysis of correlation between the mRNA level of *MAOA* and the content of E. (C) Statistical analysis of NE and E content in 35 cases of HCC tissues $^{**}p$ <0.01. (D) The expression of MAOA protein in the culture supernatant of control or MAOA overexpressed SMMC-7721 or MHCC-97L cells. GAPDH of remaining cells was used as a loading control. (E and F) SMMC-7721 (E) or MHCC-97L cells (F), pre-treated with prazosin or ICI118551, were analyzed for cell invasiveness or anoikis upon 100 nM NE stimulation. (G and H) NE-induced invasion and anoikis inhibition of SMMC-7721 (G) and MHCC-97L (H) were significantly reduced by *ADRA1A* or *ADRB2* knockdown n.s., not significant $^{*}p$ <0.05, $^{**}p$ <0.01.

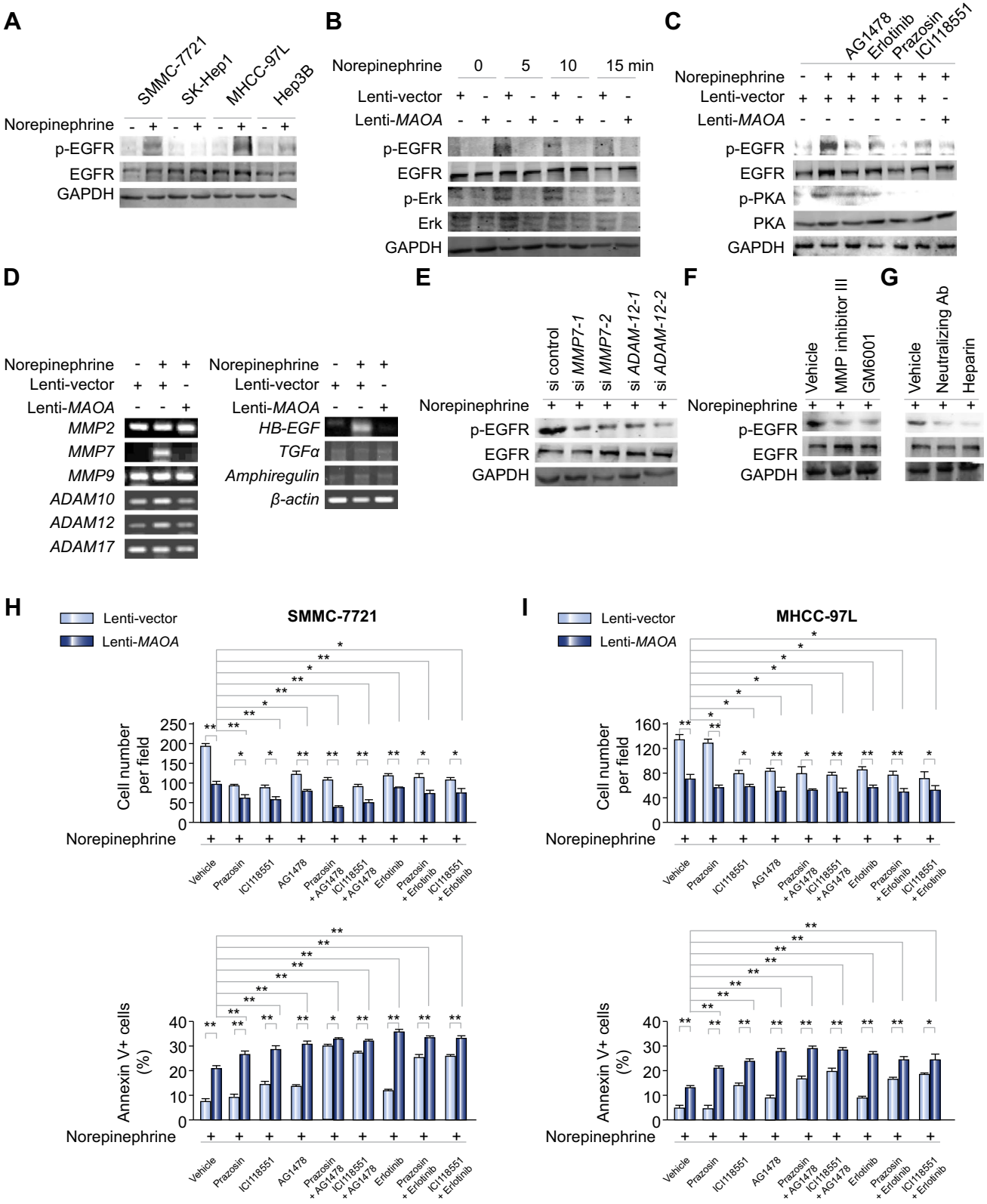
compared the levels of HB-EGF, amphiregulin, and TGF-alpha in the extracellular medium of Lenti-vector/SMMC-7721 cells and Lenti-*MAOA*/SMMC-7721 cells treated with NE at 0 min, 5 min, 10 min, and 15 min, respectively. It was found that the amount of HB-EGF in the extracellular medium was significantly higher than that of amphiregulin or TGF-alpha. Furthermore, HB-EGF release was significantly increased by NE stimulation, and decreased by MAOA overexpression (Supplementary Fig. 9A). The results also showed that the level of TGF-alpha was slightly increased by NE stimulation and was decreased by MAOA overexpression, indicating that TGF-alpha might also contribute to NE-induced EGFR transactivation (Supplementary Fig. 9A).

Furthermore, NE-induced phosphorylation of EGFR was obviously reduced by HB-EGF blockade with HB-EGF neutralyzing antibody (5 μg/ml) or heparin (100 μg/ml) (Fig. 4G). NE-induced HCC cell invasion and anoikis inhibition were also suppressed by blocking HB-EGF (Supplementary Fig. 9F and G). To further confirm that EGFR is required for NE-induced HCC cell invasion and anoikis inhibition, we knocked down EGFR with siRNA and found that the effects of NE on HCC cell invasion and anoikis was abrogated (Supplementary Fig. 10).

Taken together, these data indicated that MMP7, ADAM12, and HB-EGF are involved in NE-induced EGFR transactivation.

MAOA enhances the inhibitory effects of EGFR inhibitors or ADR antagonists on NE-induced HCC cell invasion and anoikis inhibition

The persistent release of NE from the sympathetic nerve endings that occurs during conditions of acute and chronic stress in


A
SMMC-7721
SK-Hep1
MHCC-97L
Hep3B
Norepinephrine
p-EGFR
EGFR
GAPDH
B
Norepinephrine 0 5 10 15 min
Lenti-vector
Lenti-MAOA
p-EGFR
EGFR
p-Erk
Erk
GAPDH
C
AG1478
Erlotinib
Prazosin
ICI118551
Norepinephrine
Lenti-vector
Lenti-MAOA
p-EGFR
EGFR
p-PKA
PKA
GAPDH
D
Norepinephrine
Lenti-vector
Lenti-MAOA
MMP2
MMP7
MMP9
ADAM10
ADAM12
ADAM17
HB-EGF
TGFα
Amphiregulin
β-actin
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si MMP7-2
si ADAM-12-1
si ADAM-12-2
Norepinephrine
p-EGFR
EGFR
GAPDH
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GM6001
Norepinephrine
p-EGFR
EGFR
GAPDH
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Vehicle
Neutralizing Ab
Heparin
H
Lenti-vector
Lenti-MAOA
SMMC-7721
Cell number per field
Annexin V+ cells (%)
Norepinephrine
Vehicle
Prazosin
ICI118551
AG1478
Prazosin + AG1478
ICI118551 + AG1478
Erlotinib
Prazosin + Erlotinib
ICI118551 + Erlotinib
I
Lenti-vector
Lenti-MAOA
MHCC-97L
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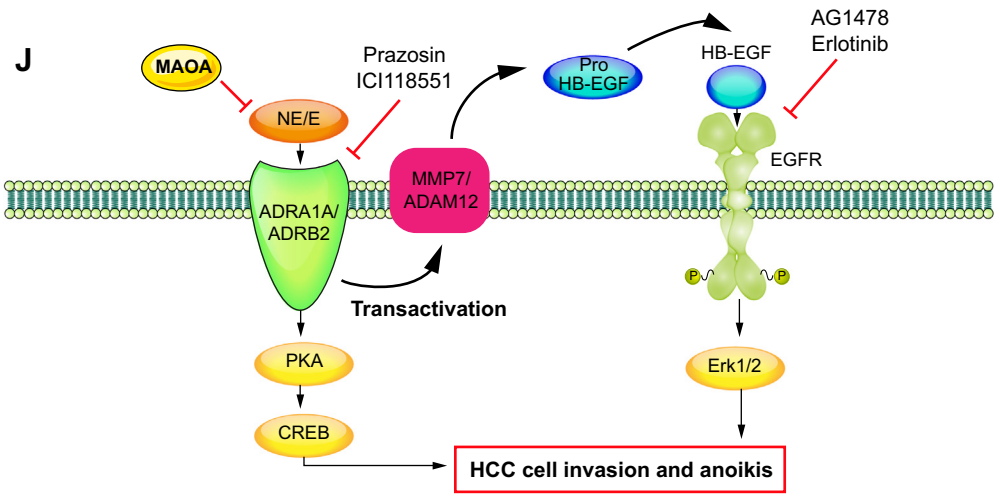

Fig 4. *(continued)*

Table 3. cDNA microarray data of MMP7/ADAM12/HB-EGF for Lenti-vector/SMMC-7721 treated with vehicle, Lenti-vector/SMMC-7721 treated with NE and Lenti-*MAOA*/SMMC-7721 treated with NE, respectively.

Symbol	AVG signal value			Fold change		
	Lenti-vector	Lenti-vector + NE	Lenti-*MAOA* + NE	Lenti-vector + NE /lenti-vector	Lenti-*MAOA* + NE/ lenti-vector + NE	Lenti-*MAOA* + NE/ lenti-vector
MMP2	0.01	0.01	0.01	1	1	1
MMP7	51.73271	67.87345	31.92715	1.31	0.47	0.62
MMP9	0.01	0.01	0.01	1	1	1
ADAM10	119.0204	136.3069	122.3337	1.15	0.90	1.03
ADAM12	4.338587	21.11914	8.095791	4.87	0.38	1.87
ADAM17	62.67328	82.16988	65.19996	1.31	0.79	1.04
HB-EGF	51.6576	72.02672	52.62322	1.39	0.73	1.02
amphiregulin	0.01	0.01	0.01	1	1	1
TGFα	0.01	0.01	0.01	1	1	1

humans (particularly cancer patients), in addition to the decreased MAOA expression that occurs in HCC, leads to the aberrant accumulation of NE in HCC tissues, which may decrease the efficiency of EGFR-targeting drugs or ADR antagonists during HCC treatment. We speculate that, by removing NE from the HCC microenvironment, MAOA may enhance the effects of EGFR inhibitors or ADR antagonists during HCC. The results indicated that NE-induced HCC cell invasion and anoikis inhibition can be suppressed by single usage of EGFR inhibitors or ADR antagonists or MAOA overexpression in SMMC-7721 cells (Fig. 4H) and MHCC-97L cells (Fig. 4I), which was synergistically suppressed by combined use of EGFR inhibitors or ADR antagonist with MAOA overexpressing, compared with that obtained in untreated transfected cells (Fig. 4H and I).

Fig. 4. MAOA inhibits ADRA1A/ADRB2-mediated transactivation of EGFR signaling, and enhances the inhibitory effects of EGFR inhibitors or ADRs antagonists on NE-induced HCC cell invasion and anoikis inhibition. (A) Western blotting analysis of phosphorylation of EGFR in four different HCC cells treated with NE for 5 min. (B) Phosphorylation of EGFR, Erk1/2 in MAOA overexpressed and control SMMC-7721 cells treated with NE for 0, 5, 10, and 15 min. GAPDH was detected as the loading control. (C) Phosphorylation of EGFR or PKA in SMMC-7721 cells treated with prazosin, ICI118551, AG1478, erlotinib and/or NE. (D) PCR analysis of *MMP7*, *ADAM12*/*HB-EGF*, *TGF-α*, and *amphiregulin* for Lenti-vector/SMMC-7721 treated with vehicle, Lenti-vector/SMMC-7721 treated with NE and Lenti-*MAOA*/SMMC-7721 treated with NE. (E and F) Phosphorylation of EGFR induced by NE was obviously suppressed by blocking MMP7 or ADAM12 with siRNA (E) or MMP inhibitor III (MMP7 inhibitor) and GM6001 (MMP7/ADAM12 inhibitor) (F). (G) Phosphorylation of EGFR induced by NE was obviously suppressed by blocking HB-EGF with anti-HB-EGF neutralyzing antibody and heparin. (H and I) EGFR inhibitors or ADRs antagonists abrogated NE-induced HCC cell invasion and anoikis inhibition of SMMC-7721 (H) and MHCC-97L cells (I) with MAOA overexpression $^{*}p$ <0.05, $^{**}p$ <0.01. (J) Model for NE/E-derived and ADRs-mediated signalings underlying HCC cell invasiveness and anoikis. Upon stimulation by NE/E, ADRA1A/ADRB2 activates MMP7/ADAM12, release HB-EGF to transactivate EGFR and downstream Erk1/2 signaling, thereby promote cell invasiveness and anti-anoikis. ADRA1A/ADRB2-mediated transactivation of EGFR signaling can be blocked by EGFR inhibitors, ADRs antagonists or MAOA overexpression. Combined use of them obtains a better inhibitory effect on ADRs-mediated transactivation of EGFR. Additionally, canonical PKA and CREB signaling also participate in this mechanism.

These data indicated that MAOA enhances the inhibitory effects of EGFR inhibitors or ADR antagonists on NE-induced HCC cell invasion and anoikis inhibition (Fig. 4J).

Discussion

As a catecholamine neurotransmitter degrading enzyme, MAOA has long been thought to be associated with human emotional and mental states. However, its functional roles in cancer development have rarely been studied. Several studies on prostate cancer (PCa) had suggested that upregulated MAOA in PCa cells was positively related to PCa progression [17,18]. On the contrary, in cholangiocarcinoma, MAOA was suppressed by the coordinated control of promoter hypermethylation and IL-6 signaling, and MAOA expression was negatively associated with cancer invasiveness [19]. These studies suggest the regulation and function of MAOA vary in different cancer types.

In HCC, DNA copy number aberrations were not found in Xp11.3, on which MAOA is located [24]. In our research, we demonstrated that MAOA expression was silenced in HCC by epigenetic methylation and histone acetylation and further identified MAOA as a negative regulator of HCC malignancy. To unveil the underlying mechanism of how MAOA affects HCC invasion and metastasis, comprehensive studies were performed and the data indicated that MAOA suppressed HCC cell invasion and metastasis by inhibiting both NE/E-initiated canonical adrenergic signaling and ADR-mediated transactivation of EGFR signaling.

NE/E were known to exert their physiological functions primarily through α- and β-ADRs [25]. In our study, we identified that NE/E-induced HCC cell resistance to anoikis was specifically mediated by the β2-ADR, which is consistent with the results of previous ovarian cancer studies [9]. However, NE/E-induced cell invasion can be mediated by both α1A- and β2-ADRs in HCC.

The adrenergic signaling pathway plays an important role in cancer progression by regulating multiple cellular processes [26–28]. Ligation of the β-ADR by NE/E has been reported to stimulate the synthesis of cyclic 3′-5′ adenosine monophosphate (cAMP) from adenylyl cyclase, which then activates protein kinase A (PKA), a cAMP effector, and eventually leads to the phosphorylation of transcription factors, such as the cAMP Response Element Binding Protein (CREB) [26,29,30]. Moreover, in the cardiovascular system, ADRs can transactivate EGFR signaling via the proteolysis of latent ligands, such as HB-EGF, by specific MMPs or ADAMs [21,23,31–33]. In the present study, we demonstrated for the first time that the ADRA1A/ADRB2 receptors can transactivate EGFR signaling in cancer.

Because the EGFR is overexpressed in the majority of adenocarcinomas and squamous cell carcinomas, the EGFR can be targeted by selective pharmacological inhibitors that are currently being used in clinical trials. Several ongoing clinical trials are currently using β-ADR antagonists (beta-blockers) as anti-cancer drugs [34–36]. And the use of beta-blockers has been reported to prevent disease progression in breast cancers and malignant melanomas [37–39]. Here, we showed that EGFR inhibitors and ADR antagonists can inhibit NE/E-induced EGFR transactivation and HCC invasion, as well as prevent anoikis. Notably, we found that MAOA synergized with EGFR inhibitors or ADR antagonists to abrogate HCC invasion and promote anoikis (Fig. 4J). The results of our study revealed that EGFR inhibitors and ADR antagonists have superior inhibitory effects in HCC cells that have been transfected with the MAOA gene compared to the untransfected controls.

The current approach to developing drugs for the prevention and treatment of cancer is to first test the effects of the drugs on cancer cell viability *in vitro* and to then conduct *in vivo* tests in mouse xenografts or experimental tumor models in laboratory rodents [29]. However, the effects of neurotransmitters, which are powerful upstream regulators, have been completely ignored. Thus, many studies, including ours, have suggested that the neurotransmitter system should be considered to be an important factor for future drug development and drug testing during clinical trials.

Taken together, we described MAOA as a key player in the adrenergic system in the control of invasion, anoikis, metastasis and clinical outcomes during human HCC. The results of our study have provided insight into the application of MAOA as a novel predictor of clinical outcomes and indicate that increasing MAOA expression or enzyme activity by using small molecules or by targeting gene-delivery systems through gene transfer may be novel approaches that can be used for the treatment of HCC.

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Conflict of interest

The authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jhep.2014.02.025.

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