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HAI-2 is epigenetically downregulated in human hepatocellular carcinoma, and its Kunitz domain type 1 is critical for anti-invasive functions

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Pharmacological demethylation-based gene expression profile analysis is a useful tool to identify epigenetically silenced tumour suppressor genes. *HGF activator inhibitor 2 (HAI-2)*, a serine protease inhibitor, has been identified as one of the candidate tumour suppressor genes in human hepatocellular carcinoma (HCC) with this technique. In this study, we aimed to characterise the epigenetic status and tumour suppressive function of *HAI-2* in HCC. We validated that *HAI-2* expression was either absent or low in most of the HCC cell lines tested, and 5-Aza-2′-deoxycytidine treatment significantly restored its expression in 9 (75%) of these 12 cell lines. *HAI-2* was found to be frequently underexpressed in human HCCs ($p < 0.001$). With bisulphite DNA sequencing and methylation-specific PCR, we found that the promoter of the *HAI-2* gene was frequently hypermethylated in both HCC cell lines and human HCCs. Ectopic expression of *HAI-2* significantly inhibited cell migration and invasiveness of HCC cells *in vitro* and suppressed tumourigenicity *in vivo*. In addition, we also provided the first evidence that HAI-2 mediated its tumour suppressor function via the Kunitz domain 1 (KD-1), as KD-1 but not KD-2 inactivating mutant abolished its anti-tumour invasiveness *in vitro*. Our findings suggest that *HAI-2* is a candidate tumour suppressor gene that is frequently hypermethylated and underexpressed in human HCCs, and the KD-1 domain of HAI-2 is the key region responsible for its anti-invasive function.

Key words: HAI-2; epigenetics; HCC; serine protease inhibitor; Kunitz domain

Hepatocellular carcinoma (HCC) is the sixth most common cancer worldwide[1] and is particularly prevalent in Southeast Asia. Epigenetic alteration is one of the major mechanisms in the inactivation of tumour suppressor genes in different cancer types including HCC.[2,3] We previously reported that epigenetic gene expression profiling using oligonucleotide microarray was an effective tool to identify epigenetically silenced tumour suppressor genes.[4] With this technique, we have identified that the *hepatocyte growth factor activator inhibitor 2 (HAI-2)* gene, which encodes a Kunitz-type serine protease inhibitor protein, was one of the genes epigenetically silenced in HCC cells.

HAI-2 is a transmembrane protein with 2 Kunitz domains on its extracellular region, namely, Kunitz domain 1 (KD-1) and Kunitz domain 2 (KD-2) (Fig. 1). It inhibits the formation of active hepatocyte growth factor (HGF) by inhibiting the activities of hepatocyte growth factor activator (HGFA).[5,6] In addition, HAI-2 inhibits a wide variety of serine proteases including trypsin, plasmin, tissue and plasma kallikreins, factor XIa and hepsin.[7–10] Some of these serine proteases are suggested to play a role in the degradation of extracellular matrix and have been implicated in tumour progression.[11–13] *HAI-2* has been found to be underexpressed in several types of cancers such as breast cancer,[14] renal-cell carcinoma,[15,16] and glioblastoma.[17,18] Ectopic expression of *HAI-2* was able to suppress tumour cell growth *in vitro* in renal-cell carcinoma[16] and inhibit cell migration and/or invasion in breast and renal-cell carcinoma.[16,19] However, the roles of HAI-2 in human HCC have not been fully characterised. In this study, we aimed to investigate the molecular mechanisms and functional roles of *HAI-2* in HCC.

Our results showed that *HAI-2* was frequently hypermethylated and underexpressed in human HCCs. It inhibited cell motility and invasiveness of SMMC-7721 HCC cells *in vitro* and suppressed HCC tumour growth *in vivo*. We further provided the first evidence that HAI-2 mutant with inactivating point mutation on KD-1 but not KD-2 domain lost its ability in suppressing migration and invasion of HCC cells. Our findings suggest that *HAI-2* inactivation is implicated in hepatocarcinogenesis and KD-1 domain of HAI-2 is the key domain responsible for the anti-tumour invasive functions.

Material and methods

Patient samples

Paired samples of human HCCs and their corresponding non-tumourous liver tissues from patients who had had surgical resection between 1992 and 2000 at Queen Mary Hospital, The University of Hong Kong, were randomly selected for this study. All specimens were obtained immediately after surgical resection, snap-frozen in liquid nitrogen, and kept at −80°C. Patient characteristics are summarised in Table I.

Plasmids

Full length human *HAI-2* cDNA was purchased from German Resource Centre for Genome Research (Berlin, Germany). *HAI-2 WT* was subcloned into pcDNA3.1+/Hygro vector (Invitrogen, Gaithersburg, MD). With reference to a previous mutation study on *HAI-1*,[20] *HAI-2* mutants with inactivation of the Kunitz domain(s) was constructed by mutating the P1 residues of the active site of the KD-1 (Arg48) and/or KD-2 (Arg143) to Ala using QuickChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA), named as *HAI-2 KD-1/2*, *HAI-2 KD-1* and *HAI-2 KD-2*.

Cell lines

HCC cell lines were obtained from the American Type Culture Collection (Manassas, VA) and the Shanghai Institute of Cell Biology (BEL-7402 and SMMC-7721). H2P and H2M cells are gift from Dr. XY Guan.[21] HCC cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS) and 100 units each of penicillin and streptomycin (Invitrogen).

Stable clones were established by transfecting 2 μg of *HAI-2* wild type and mutant constructs into SMMC-7721 cells on 6-well

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Additional Supporting Information may be found in the online version of this article.

Abbreviations: 5-Aza-dC, 5-Aza-2′-deoxycytidine; ECM, extracellular matrix; HAI, hepatocyte growth factor activator inhibitor; HCC, hepatocellular carcinoma; HGF, hepatocyte growth factor; KD, Kunitz domain; MMP, metalloprotease; MSP, methylation specific-PCR; SP, serine protease; WT, wild type.

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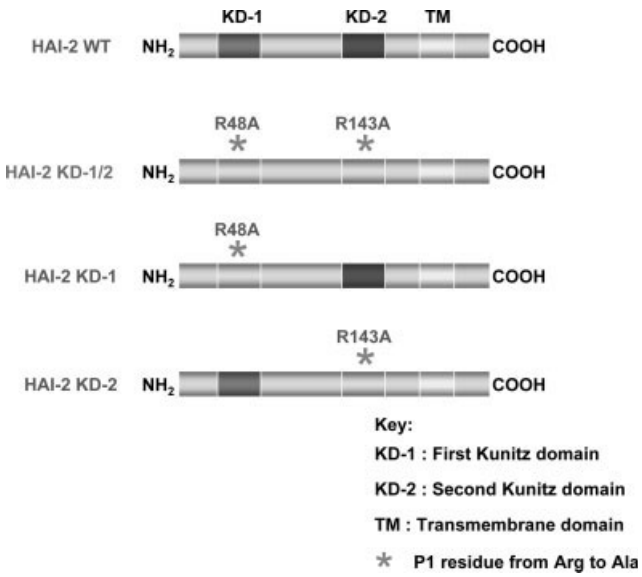


FIGURE 1 – Schematic diagram showing functional domains of HAI-2. HAI-2 belongs to the family of transmembrane Kuntiz-type serine proteinase inhibitor with a short cytoplasmic tail. HAI-2 contains 2 Kunitz domains named as KD-1 and KD-2 on its extracellular region of the protein.

plate using FuGENE 6 transfection reagent (Roche Molecular Biochemicals, Indianapolis, IN). After 24 hr, transfected cells were trypsinised and replated onto 100-mm culture dishes at 1:10 dilution and then grown in selection medium with 200 μg/ml hygromycin B (Invitrogen) for 4 weeks. Hygromycin-resistant colonies were picked and expanded. Stable expression of HAI-2 in total cell lysate was confirmed by western blotting.

5-Aza-2′-deoxycytidine (5-Aza-dC) and trichostatin A (TSA) treatment

The demethylating agent 5-azadeoxycytidine (5-Aza-dC) (Sigma, UK) was used to block DNA methylation. Cells were treated with 5-Aza-dC either at 10 μM or indicated concentration for 48 hr or for indicated durations. Drugs and culture medium were refreshed every day during treatment. Histone deacetylase inhibitor TSA (Sigma) was applied to examine whether histone deacetylation was involved in gene silencing. Cells were treated with 0.5 μg/ml of TSA for 24 hr.

Semiquantitative and quantitative real-time RT-PCR

Total RNA was extracted from HCC cell lines and human samples by TRIzol reagent (Invitrogen). First-strand cDNA was synthesized from 1 μg of total RNA using random hexamers with GeneAmp RNA PCR Kit (Applied Biosystems, Foster City, CA). The cDNA was then used for detecting the expression levels of *HAI-2* using the following primers: HAI-2 F1 (5′-AAGAA TACTGCACCGCCAAC-3′) and HAI-2 R1 (5′-TTCTTCAC CAGCTGCTCCTT-3′). Quantitative real-time PCR was performed in ABI Prism 7700 system with TaqMan Gene Expression Assays (Applied Biosystems). Experiments were done in triplicate and the expression of *HAI-2* in paired HCC and non-tumourous liver tissues from the same patient was expressed as tumour/non-tumour (T/NT) ratio. A T/NT ratio lesser or equal to 0.5 was defined as underexpression in tumour tissue.

Bisulphite sequencing and methylation-specific PCR (MSP)

Sodium bisulphite treatment was performed with the CpGenome DNA modification kit (Chemicon, Temecula, CA). Forty nanograms of bisulphite-treated DNA was used as template for PCR amplification by using the following primers: HAI-2 BS_F3 (5′-ATTAAGTTTAAGGGAAGGGTGGTAG-3′) and HAI-2 BS_R1 (5′-ATACCTAAATCTACTCCTCACTC-3′). PCR products were cloned into pGEM-T Easy vector (Promega, Madison, WI) and at least 5 individual clones were selected for sequencing. Methylation-specific PCR was performed with methylation status-specific primer pairs. They are HAI-2 MF1 (5′-AGTAGATT TAGGTATCGCGCGT-3′) and HAI-2 MR1 (5′-TAAAAT CTCGCGATCAAATACG-3′) for methylated alleles and HAI-2 UF1 (5′-GAGTGAGGAGTAGATTTAGGTATTGTGT-3′) and HAI-2 UR1 (5′-TTAAAATCTCACAATCAAATACAAA-3′) for unmethylated alleles. Bisulphite-treated DNA obtained from *in vitro*-methylated DNA (Chemicon) and placenta DNA was used in each batch of PCR analysis as methylated and unmethylated controls, respectively.

TABLE I – SUMMARY OF THE CLINICOPATHOLOGICAL CHARACTERISTICS OF HCC PATIENTS

	No. of patients (%)
Age	
Mean (range)	54.1 years (27–74 years)
Sex	
Male	38 (76)
Female	12 (24)
Serum HBsAg	
Positive	40 (80)
Negative	10 (20)
Nontumourous livers	
Non-cirrhotic	25 (50)
Cirrhotic	25 (50)
Tumour Stage	
I–II	17 (34)
III–IV	30 (60)
Cellular differentiation (Edmondson's grading)	
I–II	20 (40)
III–IV	30 (60)
Tumour size (cm)	
<5	18 (36)
>5	32 (64)

Western blot analysis

Total protein from cultured cell lines was extracted in RIPA lysis buffer (50 mM, pH 7.4 Tris-HCl, 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA, 5 mmol/L sodium fluoride) with freshly added Complete EDTA-free Protease Inhibitor Cocktail Tablets (Roche). Twenty micrograms samples was loaded in each lane for SDS-PAGE and then transferred to polyvinylidene difluoride membrane for immunoblotting. The expression of HAI-2 protein was detected by mouse monoclonal antibody against human HAI-2 at 1:1000 dilution (R&D Systems, Minneapolis, MN) and anti-mouse HRP antibody against mouse IgGs at 1:5000 dilution (GE Healthcare Life Sciences, Piscataway, NJ).

In vitro *cell migration and invasion assays*

Cell migration assay was performed using transwell inserts with polycarbonate membranes, 8.0-μm pore size (Corning, NY). Matrigel invasion assay was done using extracellular matrix (ECM) Cell Invasion Assay kit (Chemicon). In brief, 1×10^5 cells in serum-free medium were seeded onto the upper chamber. Culture medium containing 10% FBS was used as a chemoattractant in the lower chamber. Cells were incubated in a humidified incubator at 37°C for 12 (migration assay) or 24 hr (invasion assay). Migrated/invaded cells on the lower surface of the membrane were then fixed and stained with crystal violet. Stained cells in each field was photographed and counted in 5 fields. Each experiment was performed thrice.

In vivo *tumourigenicity assay*

To assess the tumourigenicity, HAI-2 and its mutant stably transfected cells were trypsinised and resuspended in phosphate-

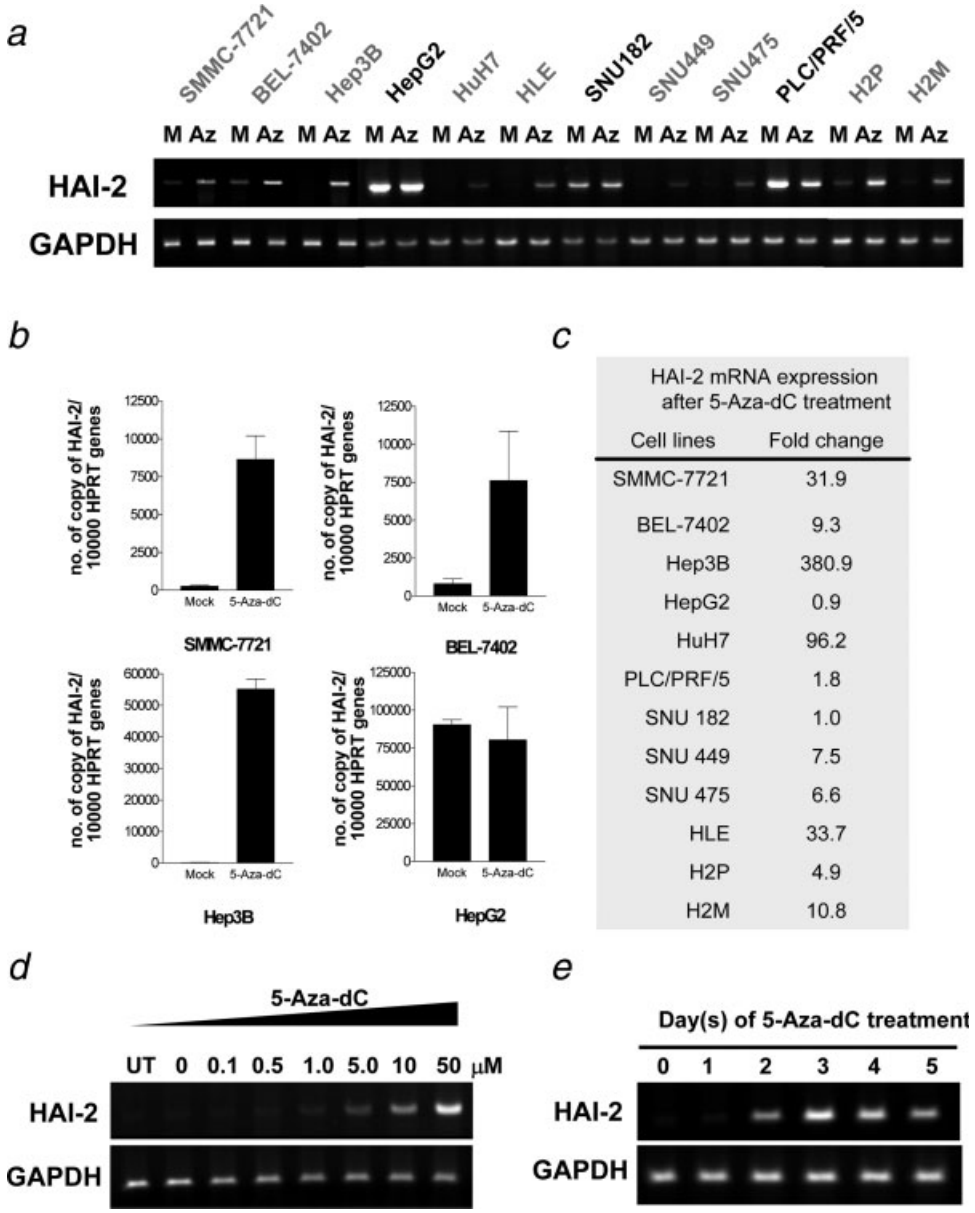


Figure 2 – Restoration of *HAI-2* expression in human HCC cell lines upon 5-Aza-dC treatment. (*a*, *b*) With both semi- and real time-quantitative PCR, 9 of the 12 HCC cell lines showed increased expression of *HAI-2* mRNA upon treatment with 5-Aza-dC. (*c*) Fold changes of increased *HAI-2* expression with real time-quantitative PCR. (*d*) Different concentrations of 5-Aza-dC were applied to SMMC-7721 HCC cell line. 5-Aza-dC induced expression of *HAI-2* in a dose-dependent manner. (*e*) The expression of *HAI-2* in SMMC-7721 treated with 10 μM 5-Aza-dC was also in a time-dependent manner. M, mock; Az, 5-Aza-dC; UT, Untreated control.

buffered saline. Cells (1×10^6) were injected subcutaneously into the flank of 6-week-old male BALB/c nude mice using a 25-gauge needle ($n = 5$ for each group of experiments). Tumour size was monitored weekly by measuring the largest and smallest diameters of tumour and estimated according to the formula: volume = 1/2 × (largest diameter) × (smallest diameter).[2] This experiment was preformed following the Animals (Control of Experiments) Ordinance (H.K.) and Institute's guidance on animal experiments.

Statistical analysis

HAI-2 expression level between tumours and non-tumourous liver tissues were compared with Mann–Whitney test using SPSS for Windows 14.0 (SPSS, Chicago, IL). Results from migration and invasion assays were analysed using Student's *t*-test. All test results were considered significant when p values were less than 0.05.

Results

Restoration of HAI-2 expression upon treatment with 5-Aza-dC

Our previous study using epigenetic gene expression profiling has identified *HAI-2* as epigenetically silenced gene in 3 HCC cell lines.[4] In this study, we validated the promoter methylation and analysed the expression level of *HAI-2* in HCC cell lines and human HCCs.

Upon treatment with 10 μM 5-Aza-dC for 4 days, the mRNA expression level of *HAI-2* was found to be upregulated in most of the 12 HCC cell lines, with the exception of HepG2, SNU182 and PLC/PRF/5 (Fig. 2*a*). The restored expression of *HAI-2* was fur-

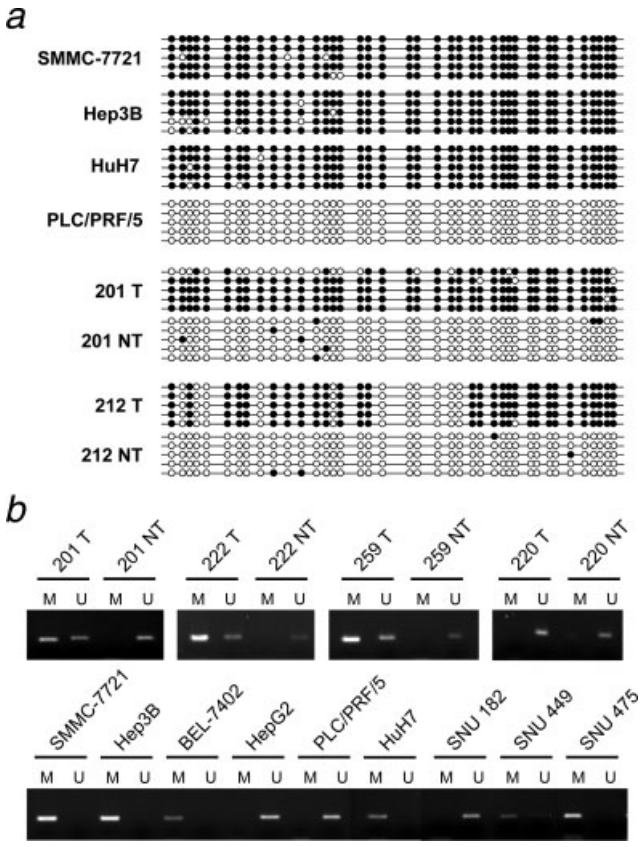


Figure 3 – The methylation pattern of *HAI-2* promoter using bisulphite sequencing and MSP. (*a*) The promoter of *HAI-2* was highly methylated in HCC cell lines including SMMC-7721, Hep3B, and HuH7 and in human HCCs as compared with corresponding non-tumourous liver tissues. (*b*) MSP primers were designed based on the promoter sequence of *HAI-2*. The promoter of *HAI-2* was methylated in most of the HCC cell lines. In human HCCs, 76% (38/50) showed promoter methylation in the tumours but not in the corresponding non-tumourous livers. Solid circle, methylated CpG; Open circle, unmethylated CpG; M, methylated allele; U, unmethylated allele; T, HCC; NT, non-tumourous liver tissue.

ther confirmed by real-time quantitative RT-PCR (Figs. 2*b* and 2*c*). Moreover, 5-Aza-dC induced *HAI-2* expression in a dose-dependent (Fig. 2*d*) and time-dependent manner (Fig. 2*e*) in SMMC-7721 cells. The earlier findings indicate that *HAI-2* was epigenetically silenced in HCC cell lines.

Frequent promoter hypermethylation of HAI-2 in HCC cell lines and human HCCs

The methylation status of *HAI-2* promoter was examined by bisulphite sequencing. The *HAI-2* promoter was highly methylated in HCC cell lines, including SMMC-7721, Hep3B, and HuH7 (Fig. 3*a*). In contrast, the *HAI-2* promoter of 5-Aza-dC unresponsive cell line PLC/PRF/5 was entirely unmethylated. The methylation status of these cell lines was consistent with their responsiveness towards 5-Aza-dC treatment (Fig. 2*b*). *HAI-2* promoter was highly methylated in human HCCs but mostly unmethylated in their corresponding non-tumourous livers (Fig. 3*a*). These observations have provided strong evidence supporting the notion that DNA methylation is implicated in *HAI-2* inactivation in human HCC.

To evaluate the *HAI-2* methylation status in a larger collection of human HCCs and their corresponding non-tumourous livers, we employed MSP. Primers for MSP were designed and their specificity was confirmed by *in vitro* methylated and normal placental DNA (Supporting Fig. S1). In HCC cell lines, the methylation status revealed by MSP was greatly consistent with the findings obtained from bisulphite sequencing (Fig. 3*b*). Most of the HCC cell lines were highly methylated in their *HAI-2* promoter except HepG2, SNU 182 and PLC/PRF/5 cells. Furthermore, 76% (38/50) of the human HCCs showed *HAI-2* promoter methylation in the tumour tissues but not in their corresponding non-tumourous livers (Fig. 3*b*). Altogether, these findings validated that *HAI-2* methylation was frequent in both HCC cell lines and human HCCs.

To demonstrate a direct effect between restoration of *HAI-2* mRNA and its methylation status, the methylation status of the *HAI-2* promoter in SMMC-7721 cells before and after 5-Aza-dC treatment at 10 μM for 4 days was examined using MSP. The amount of *HAI-2* methylated alleles of SMMC-7721 cells was greatly reduced after 5-Aza-dC treatment (Supporting Fig. S1*b*) and that of the unmethylated alleles dramatically increased as compared with that of the untreated control. Thus, demethylation of its promoter upon 5-Aza-dC treatment restored *HAI-2* expression in SMMC-7721.

HAI-2 was underexpressed in human HCCs

We further examined the expression level of *HAI-2* in HCC cell lines and human HCCs. Expression of *HAI-2* was either absent or present at low levels in most of the human HCC cell lines (Fig. 4*a*). Also, *HAI-2* mRNA was frequently underexpressed in human HCCs (80%, 16/20) when compared with their corresponding non-tumourous livers (Fig. 4*b*), using semi-quantitative RT-PCR. With real-time quantitative RT-PCR, we confirmed that *HAI-2* mRNA was frequently (68%, $n = 34$) underexpressed in the 50 HCCs as compared with their corresponding non-tumourous livers ($p < 0.001$, Mann Whitney test) (Fig. 4*c*). Comparing the results of expression level and methylation status, we found that 71% of the HCC cases with underexpression of *HAI-2* had hypermethylation in their promoters, indicating that *HAI-2* was frequently silenced by promoter hypermethylation.

Histone modification did not play a significant role in HAI-2 inactivation in HCC cells

From the earlier results, we have shown that *HAI-2* inactivation was mainly attributed to promoter hypermethylation. However, histone modification may also be involved in gene silencing and it may also associate with DNA methylation.[22,23] To examine the effect of histone deacetylation on *HAI-2* expression, we treated the HCC cell lines with TSA, a histone deacetylase inhibitor. Using real-time PCR, the mRNA expression level of *HAI-2* was found to be slightly upregulated in only 3 of the 12 HCC cell lines (Figs. 5 *a* and 5*b*), indicating that TSA had little or no effect on HAI-2 expression in HCC cell lines. Although TSA has been shown to reactivate gene transcription in a synergistic manner with 5-Aza-dC,[22,23] we failed to show that TSA synergistically enhanced 5-Aza-dC-mediated *HAI-2* expression in 3 of the HCC cell lines, including the HLE cells (Fig. 5*c*). Our observation indicates that DNA methylation, but not histone deacetylation, is involved in *HAI-2* inactivation.

HAI-2 inhibited the migration and invasion of SMMC-7721 HCC cells

We investigated whether *HAI-2* could inhibit cell migration and invasiveness of SMMC-7721 HCC cells. In cell migration assay, we observed that *HAI-2*-stably expressing SMMC-7721 cells showed a significant reduction in the number of migrated cells (43 ± 3) as compared with that of the vector control (109 ± 10) ($p < 0.001$) (Fig. 6*a*). Upon Matrigel cell invasion assay, *HAI-2*-stably expressing SMMC-7721 cells showed a significant reduction in cell invasiveness with a significant decrease in the number of invaded cells (100 ± 15) as compared with that of the vector control (168 ± 17) ($p = 0.007$) (Fig. 6*b*). Such inhibitory effects were abolished in *HAI-2 KD-1/2* with both KD-1 and KD-2 domains

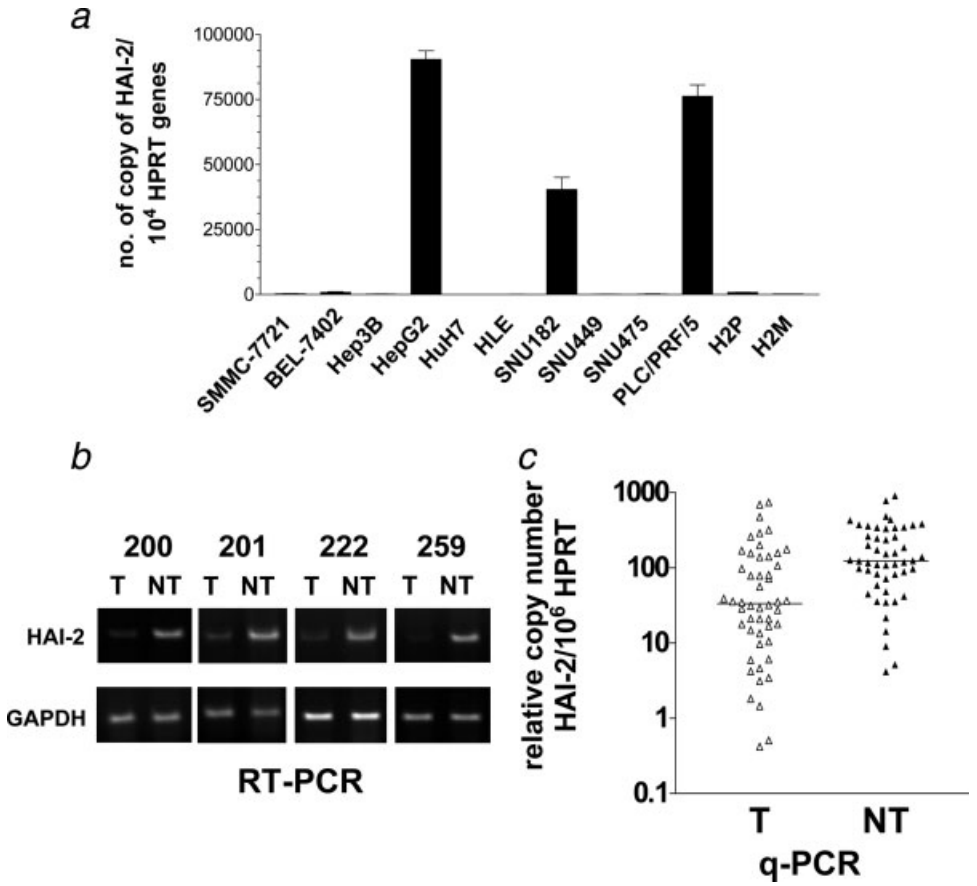


Figure 4 – Underexpression of *HAI-2* in primary human HCC cell lines and HCC samples. (*a*) Real-time quantitative PCR results. *HAI-2* was either not expressed or expressed at low levels in most of the human HCC cell lines. (*b*) Semi-quantitative PCR and (*c*) real-time quantitative PCR results. *HAI-2* was frequently (68%) and significantly underexpressed in the HCCs as compared with their corresponding non-tumourous livers ($p < 0.001$). T, HCC; NT, non-tumourous liver tissue. Horizontal line: mean expression level.

mutated and inactivated (117 ± 16 migrated cells and 138 ± 15 invaded cells, respectively) (Figs. 6*a* and 6*b*). Therefore, we have demonstrated that *HAI-2* suppressed the migration and invasion of SMMC-7721 HCC cells *in vitro*, and this suppression was abolished by the inactivating double mutations of the KD-1 and KD-2.

HAI-2 inhibited tumour growth of SMMC-7721 HCC cells in vivo

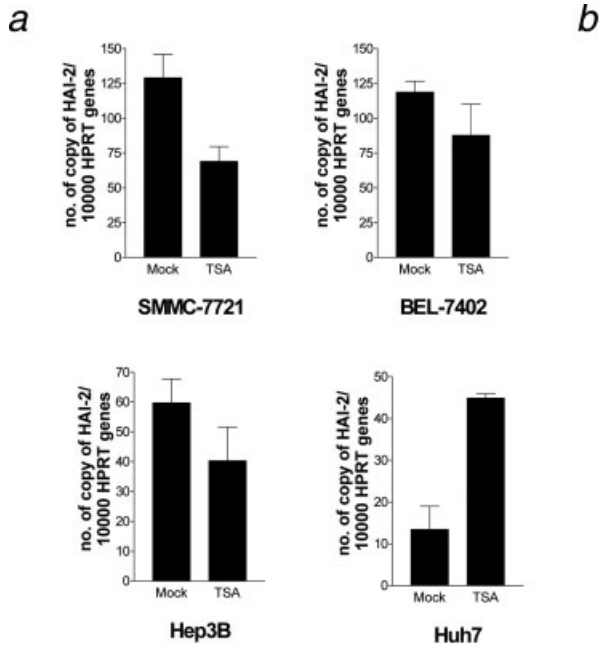
Serine proteases were found to be commonly deregulated during tumour growth and progression.[24] These prompted us to investigate the suppressive role of serine protease inhibitor *HAI-2* in tumour growth. We tested whether overexpression of *HAI-2* could suppress colony formation and cell proliferation in HCC cells and found that transient expression of HAI-2 did not suppress colony forming ability of HCC cells (Supporting Fig. S2). Furthermore, stable expression of *HAI-2* also showed no effect on HCC cell proliferation (Supporting Fig. S3). Interestingly, we observed that HAI-2 significantly suppressed HCC cell growth *in vivo* in nude mice, as indicated by a reduction in size and weight of tumours formed by HAI-2 WT stably expressing cells as compared with those of vector control (0.0092 ± 0.0070 g and 0.0522 ± 0.0306 g, respectively) ($p < 0.018$) (Figs. 6*c* and 6*d*). In contrast, HAI-2 KD-1/2 double mutant abolished this growth suppressive effect, suggesting an indispensable role of the KDs in HAI-2-mediated tumour suppression.

KD-1 was the major domain in inhibiting migration and invasiveness of SMMC-7721 HCC cells

In the aforementioned experiments, we showed that *HAI-2 KD-1/2* double mutant abolished the suppressive effect on migration and invasion of SMMC-7721 HCC cells. We queried which KD of HAI-2 was responsible for this suppression and therefore tested this with the stable KD-1 and KD-2 mutants. We observed that HAI-2 WT and HAI-2 KD-2 mutant showed significant suppression of HCC cell migration, with a significant decrease in the number of migrated cells (55 ± 4 and 22 ± 1, respectively), as compared with that of the vector control ($p < 0.001$) (153 ± 18). In contrast, KD-1 mutation abolished this suppressive effect of HAI-2 on cell migration (106 ± 7) (Fig. 7*a*). Similar results were observed in the cell invasion assay, in which the number of invaded HAI-2 KD-1 mutant cells (108 ± 13) was significantly higher than that of HAI-2 WT and HAI-2 KD-2 clones ($p = 0.048$ and $p < 0.001$) (83 ± 9 and 29 ± 2, respectively) (Fig. 7*b*). Our results demonstrated that the KD-1 domain of HAI-2 was indispensable in inhibiting the migration and invasion of SMMC-7721 HCC cells.

Discussion

In this study, we documented that *HAI-2* was frequently and significantly underexpressed in both HCC cell lines and human HCCs. In addition, DNA methylation of its promoter was very frequent in both HCC cell lines and human HCCs. We were able to demonstrate that the restoration of *HAI-2* expression in SMMC-7721 upon 5-Aza-dC treatment was directly related to the demethylation of its promoter. Moreover, 71% of our HCC cases with underexpression of *HAI-2* had hypermethylation in their promoters, indicating that *HAI-2* was frequently silenced by promoter hypermethylation. The results were consistent with our previous



b

HAI-2 mRNA expression after TSA treatment	
Cell lines	Fold change
SMMC-7721	0.5
BEL-7402	0.7
Hep3B	0.7
HepG2	1.4
HuH7	3.3
PLC/PRF/5	0.9
SNU 182	1.1
SNU 449	1.1
SNU 475	2.3
HLE	2.6
H2P	0.3
H2M	0.3

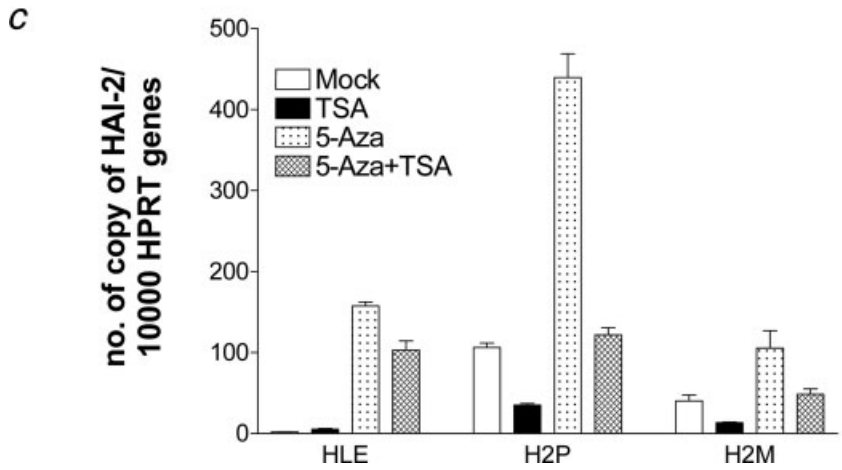


FIGURE 5 – Effect of histone deacetylation on *HAI-2* expression. (*a*, *b*) TSA treatment. The levels of *HAI-2* expression as assessed by real time-quantitative PCR and their fold changes in HCC cell lines upon TSA treatment at 0.5 μg/ml for 24 hr are shown. The *HAI-2* expression was not increased upon TSA treatment in most of the 12 HCC cell lines, except for 3 (HuH7, SNU 475 and HLE) showing mildly increased expression of *HAI-2* mRNA upon treatment. (*c*) 5-Aza-dC/TSA treatment. Three HCC cells were treated with 5-Aza-dC at 0.5 μM for 48 hr with or without TSA at 0.5 μg/ml for 24 hr. The expression of *HAI-2* mRNA was then determined by real-time-quantitative PCR. TSA did not synergistically enhance the 5-Aza-dC-mediated HAI-2 expression.

preliminary finding from epigenetic gene expression profiling using oligonucleotide microarray.[4] This supports that pharmacological demethylation-based gene expression profile analysis is a useful tool to identify epigenetically silenced tumour suppressor genes. Epigenetic inactivation of *HAI-2* was also found in human cancers such as renal-cell carcinoma and glioblastoma.[16,18] Although somatic mutation of *HAI-2* gene was not investigated in this study, previous study on renal-cell carcinoma showed that somatic mutation of *HAI-2* was rare.[16] Therefore, somatic mutation of *HAI-2* may not be a common cause of *HAI-2* inactivation in human cancers. Although one recent study by Fukai *et al*. indicated that *HAI-2* was frequently hypermethylated in human HCC,[25] the functional implications of *HAI-2* inactivation in HCC have not been explored. This prompted us to further characterise the potential tumour suppressive role of *HAI-2* in HCC.

Our data showed that ectopic expression of *HAI-2* significantly reduced the cell migration and invasion of SMMC-7721 HCC cells *in vitro*. These findings are consistent with previous observations in other cancer models.[16,19] Although the molecular mechanism of HAI-2 in tumour suppression is not yet clear, it is logical to hypothesize that it may work by suppressing the proteolytic activities of several oncogenic proteases. Parr and Jiang[19] demonstrated that pro-HGF and HGFA secreted by fibroblast cells enhanced the migration and invasion of MDA-MB-231 breast cancer cells in coculture system, whilst this fibroblast-mediated breast cancer cell invasion was greatly reduced by ectopic expression of *HAI-2*. In human HCC, HGF receptor *Met* was found to be overexpressed in human HCC[26,27] but *HGF* was downregulated in tumour cells.[28,29] Therefore, we checked the expression of *HGF*, *Met*, *HGFA* and *HAI-1* in HCC cell lines using semi-quantitative RT-PCR (data not shown). Consistently, *Met* was expressed in all of the HCC cell lines and *HGF* was not expressed in most of them. *HGFA* was only expressed in some the HCC cell lines. *HAI-1*, another member of HAI family, was

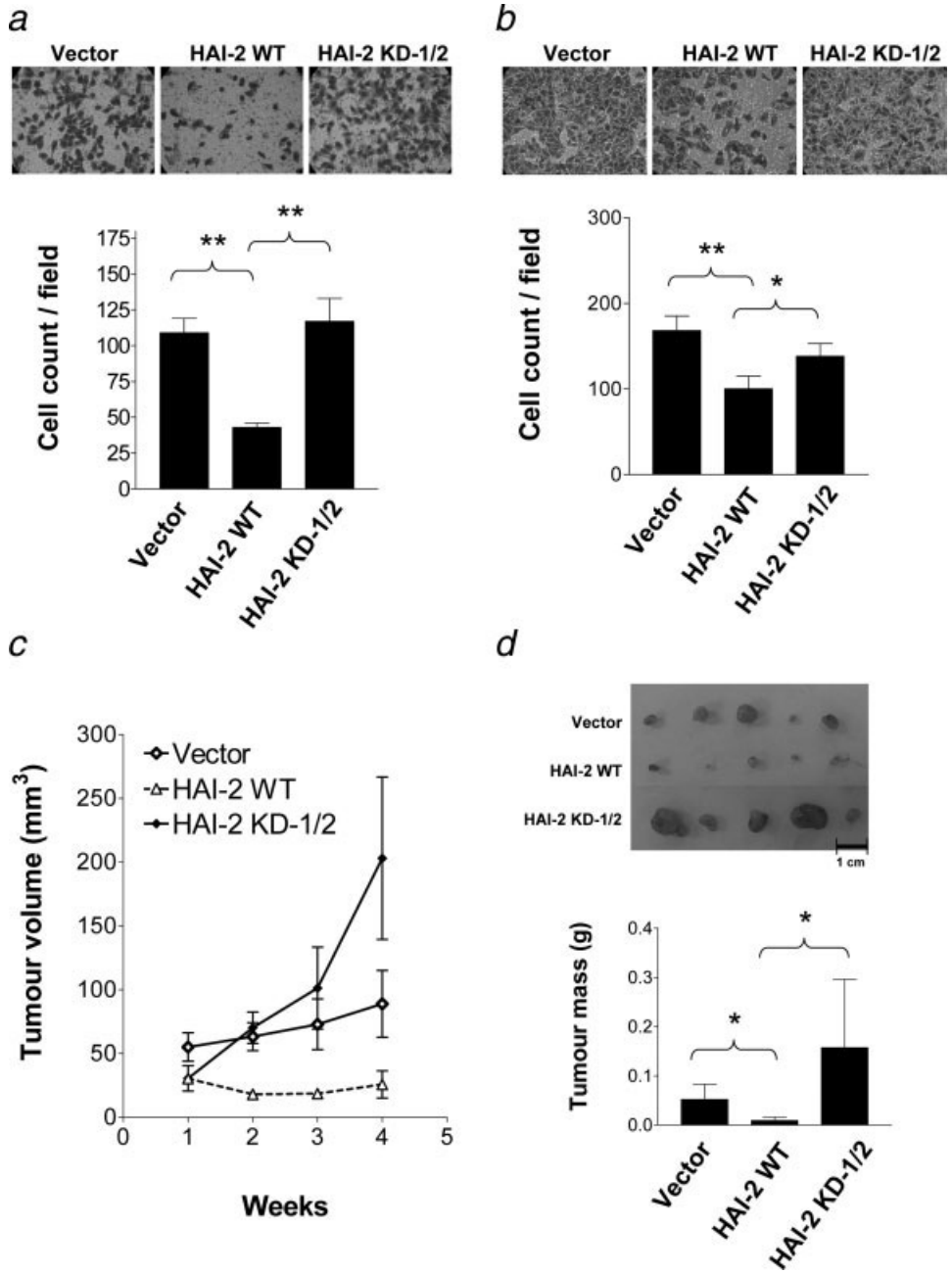


Figure 6 – Ectopic expression of *HAI-2* inhibited migration and invasion *in vitro* and tumour growth *in vivo*. (*a*) Transwell migration assay. Migrated cells of the vector control, HAI-2 WT and HAI-2 KD-1/2 were stained, photographed and counted. The number of migrated HAI-2 WT cells was significantly lower than that of vector control, whereas the number of migrated HAI-2 KD-1/2 cells was similar to the vector control. (*b*) Matrigel cell invasion assay. Empty vector, HAI-2 WT and HAI-2 KD-1/2 stably transfected SMMC-7721 cells were seeded onto the ECM-coated invasion chamber. Invaded cells were stained, photographed and counted. The number of invaded *HAI-2 WT* stably expressing cells was significantly lower than that of vector control, whereas the invasiveness of HAI-2 KD-1/2 transfectants was similar to that of the vector control. (*c*, *d*) Nude mice injection assay. The diameters of the tumours were measured regularly for 4 weeks after the injection and weighted after sacrifice. Tumours formed by HAI-2 WT expressing cells grew significantly more slowly than those of the vector control clones ($p = 0.018$). HAI-2 KD-1/2 expressing cells did not inhibit tumour growth and there was no significant difference in tumour size and tumour weights as compared with the vector control ($p = 0.187$). (*$p < 0.05$; **$p < 0.01$).

found to be underexpressed in most of the HCC cell lines. However, we observed that *HAI-2* underexpression was much more frequent than that of *HAI-1* in primary HCC (data not shown). From the expression of HGF/Met signaling molecules, we did not find any significant relationship between these HGF/Met signaling-related genes and the level of *HAI-2*. It is presumed that constitutive activation of Met receptor by overexpression or mutation is one of the main mechanisms in hepatocarcinogenesis and this hypothesis has been proven in the *Met*-transgenic mouse model.[30,31] Therefore, the role of HGF and its major regulator molecules, including HGFA and HAI-1, are minor relevance to the hepatocarcinogenesis. However, unlike HAI-1, HAI-2 can

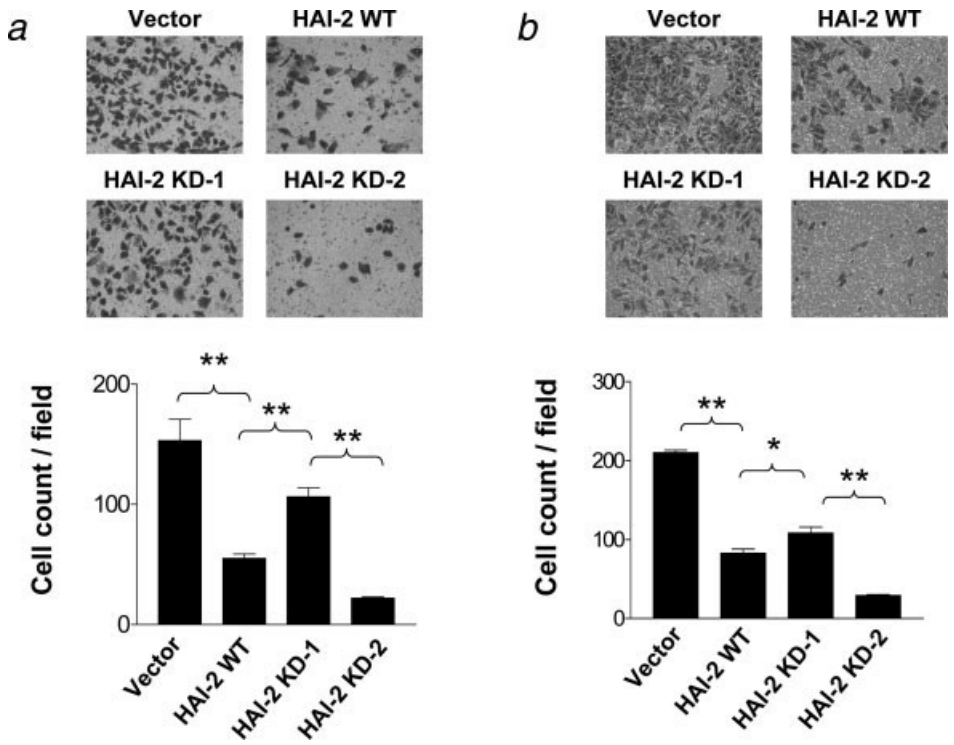


Figure 7 – Mutation of KD-1 abrogated the inhibitory effect of HAI-2 on HCC cell migration and invasion. (*a*) Transwell migration assay. The numbers of migrated HAI-2 WT and HAI-2 KD-2 stably expressed cells were comparable to one another and were significantly lower than that of vector control. However, the inhibitory effect was significantly reduced in HAI-2 KD-1 clone as compared with that of HAI-2 WT clone ($p < 0.001$). (*b*) Cell invasion assay. The numbers of invaded HAI-2 WT and HAI-2 KD-2 stably expressed cells were significantly lower than that of vector control. However, the inhibitory effect of HAI-2 KD-1 was reduced as compared with that of HAI-2 WT clone ($p = 0.048$). (*$p <$ 0.05; **$p < 0.01$).

inhibit a broad spectrum of serine proteases and therefore we hypothesize that HAI-2 may act on other serine proteases and signaling pathways to inhibit tumour growth and cell invasion. Indeed, HAI-2 can also suppress other pro-metastatic serine proteases such as hepsin.[10]

Although this manuscript was being revised, HAI-2 was reported to be a novel binding partner of matriptase-1.[32] Matriptase-1 has been implicated in tumour progression and metastasis in various types of cancers.[33] Matriptase-1 has also been found to be deregulated in breast cancer cells[34] and play a role in the progression of ovarian cancer.[35] It enhances the invasive growth of prostate and colon cancer cell lines.[36] Using tissue microarray analysis, matriptase-1 has been found to be overexpressed in human HCC.[37] Therefore, matriptase-1 may play a role in the progression of HCC. Indeed, matriptase-1 has been implicated in ECM degradation via activation of MMP-3.[38] It is tempting to speculate that HAI-2 may play an inhibitory role in regulating MMP-3 activities via matriptase-1 to prevent ECM degradation. Indeed, using the semi-quantitative PCR, we found that *matriptase-1* was expressed in SMMC-7721 (data not shown). Therefore, we hypothesize that ectopic expression of HAI-2 may act on matriptase-1 to suppress the invasion and tumour growth of SMMC-7721 cells.

Wild type *HAI-2* has been shown to play a role in tumour suppression in several cancer types.[15–17,19,25,39] Previous study has shown that KD-1 was the major functional domain in HAI-2 in inhibiting the activity of its target proteases *in vitro*.[6] However, the roles of the 2 KDs of HAI-2 in tumour suppressive functions have not been clarified. In this study, we have provided strong evidence that wild type HAI-2 as well as HAI-2 KD-2 mutant significantly inhibited *in vitro* migration and invasion of SMMC-7721 cells. However, such inhibition of tumour invasive function was abolished with inactivating mutations of HAI-2 KD-1 and HAI-2 KD-1/KD2. Therefore, our results have lent strong support that KD-1, and not KD-2, of HAI-2 is the key domain responsible for inhibiting the migration and invasion of HCC cells.

Our results revealed that HAI-2 suppressed tumour growth *in vivo* in nude mice. In addition, such inhibitory effects were abolished in *HAI-2 KD-1/2* with both KD-1 and KD-2 domains mutated and inactivated. This suggests an indispensable role of the KDs in HAI-2-mediated tumour suppression. However, such growth inhibitory effect was not observed *in vitro*. HAI-2 is a cell surface protease inhibitor and this implies that it works on the cell surface and in the extracellular environment. It is logical to speculate that HAI-2 may require interaction with stromal cells, ECM molecules in extracellular environment and other membrane proteins to inhibit tumour growth. Li *et al*. supported our hypothesis, on the study of HAI-2 targeting protease, hepsin, in prostate cancer model. They showed that the growth promoting effect of hepsin-stably transfected prostate cancer cells was only observed in animal model but not in cell culture model.[40] These observations raise the possibility that proteases and their inhibitors may regulate tumour growth via the interaction of the extracellular environment.

In conclusion, our study has shown that *HAI-2* was frequently inactivated in human HCC and this was attributed to promoter hypermethylation. Ectopic expression of *HAI-2* suppressed cell migration, invasiveness and tumour growth of HCC cells. KD-1 of HAI-2 is implicated to be the key domain responsible for its tumour invasive functions. We have also documented that the suppressive role of HAI-2 in tumour growth was significant only *in vivo*, implying the importance of extracellular environment for its function.

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