*Original Article*

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# Expression of ankyrin repeat and SOCS box containing 4 (ASB4) confers migration and invasion properties of hepatocellular carcinoma cells

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**Summary** **Ankyrin repeat and SOCS box containing 4 (ASB4) involves in physiological process of ubiquitin-mediated proteasomal degradation. Our previous study demonstrated high expression of ASB4 in hepatocellular carcinoma (HCC) cell lines. This study further reveals its clinical implications and tumorigenic properties in HCC. Analysis of 217 HCC gene expression profiles followed by validation in a separate cohort of 50 cases illustrated high ASB4 in HCC. Among the 50 cases, 54% of tumors exhibited more than 2-fold up-regulation of ASB4. Elevated ASB4 associated with low serum level of a HCC serological marker alpha-fetoprotein (AFP), postulating of its use to differentiate AFP-negative HCC. Suppression of ASB4 in PLC and MHCC97-L HCC cells hindered the cell migration and invasion. Reciprocally, enhanced migration rate was measured when ASB4 was ectopically expressed in Hep3B HCC cells. Cross comparison of results derived from *in silico* predictions of seed-matched sequences and by analyzing human HCC databases with matched microRNA and gene expression profiles, microRNA-200 (miR-200) family members including miR-200a and miR-200b were predicted to regulate ASB4 expression in HCC. MiR-200a showed inversed expression level with ASB4 in several of studied HCC cell lines. Dual luciferase reporter assay confirmed the presence of miR-200a binding site on the 3' untranslated region of ASB4. Reduced ASB4 level was noticed under the influence of miR-200a mimic treatment, for which this mimic-induced effect was neutralized with miR-200a inhibitor. In conclusion, this study demonstrates for the first time on the involvement of ASB4 in HCC and that its level is regulated by miR-200a.**

***Keywords:*** HCC, ASB4, migration, miR-200a

## 1. Introduction

Hepatocellular carcinoma (HCC), a major type of primary liver malignancy, ranks fifth as the most frequently diagnosed cancer in men worldwide with more than half a million new cases annually (*1*). Though with evolving clinical strategies including radioembolization and high-intensity focused ultrasound to treat this cancer, current management of HCC still rely heavily on tumor resection and liver transplantation (*2-4*). Prognosis of HCC patients remains suboptimal especially for those patients not amendable to currently available treatments. Unlike other solid tumors with a portion of patients reacting to chemotherapeutic drugs or targeting therapies, HCC is highly resistant in nature and not responding well to most chemotherapeutic agents. Due to the heterogeneity of HCC as revealed by genome-wide studies performed in Asian cohort (*5*), the use of molecular targeting drug is limited at this stage. For the clinically-approved drug sorafenib, the response rate is low and survival benefit remains modest (*6*). Not accounting strong drug resistance property of HCC, liver tumors behave similarly as other cancers by undergoing active proliferation, division and differentiation when progress from early to advanced stage. These properties

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can somehow be found during normal development in the process of embryogenesis. By knowing their presence in embryonic livers and HCC, a group of oncofetal molecules have been studied. Some of them like SALL4, glypican-3, and eIF5A2 have potential use for cancer diagnosis and treatment (*7-9*). Ankyrin repeat and SOCS box containing 4 (ASB4) is one such molecule previously identified by our team to have expression in fetal livers and cultured HCC cells (*10*).

ASB4 belongs to the ASB family, for which most of its members possess two structural domains, the ankyrin repeat and the SOCS box. The presence of these domains enables ASB proteins to act as an adaptor linking other subunits of the E3 ubiquitin ligase complex, and at the same time facilitates the binding of target protein for ubiquitination and subsequent proteasomal degradation (*11-13*). Aside its involvement in ubiquitous protein degradation, ASB4 also involves in vascular system. During endothelial development, factor inhibiting hypoxia-inducible factor 1 (FIH) can hydroxylate ASB4 under oxygen tension, thereby promoting angiogenesis (*14*). A later study has then revealed the interaction of ASB4 and nuclear factor-κB (NF-κB) in endothelial cells (*15*). Other than these roles, ASB4 functions in nervous system by up-regulating the expression of proopiomelanocortin (POMC) gene in neurons in mice (*16*). Despite all these studies on ASB4 in normal physiology, no investigation has been performed so far to uncover the role of ASB4 in cancers. In continuation of our previous proteomic study demonstrating the link between ASB4 and liver cancer (*10*), this study further examines the expression and tumorigenic properties of ASB4 in HCC. Lastly, the level of ASB4 is revealed subjected to microRNA (miRNA)-based regulation.

## 2. Materials and Methods

### 2.1. *Cell culture*

A panel of human liver cell lines composing of immortalized normal hepatocytes MIHA and HCC cells, PLC/PRF/5 (PLC), HepG2, Hep3B, MHCC97-H (97H), MHCC97-L (97L), H2M, H2P, and Huh7, were used and cultured in Dulbecco's Modified Eagle Medium (Life Technologies, Grand Island, NY, USA) with 10% fetal bovine serum (FBS) (Life Technologies) and 1% penicillin and streptomycin (Life Technologies) (*17*).

### 2.2. *Clinical specimens*

Fifty pairs of HCC and adjacent non-tumor liver tissues were obtained from patients underwent hepatic resection or liver transplantation in Queen Mary Hospital, Hong Kong from September 2001 to June 2005. Non-tumor liver tissues were obtained at least 1 cm from the tumor margin. Tumors were classified according to the new AJCC staging system. The use of clinical specimens for research was approved by the Institutional Review Board of our institute. Informed consents were obtained from patients for the use of specimens for research.

### 2.3. *Real-time quantitative polymerase chain reaction (qPCR)*

TRIzol reagent (Life Technologies) was used to extract total RNA from cultured cells and clinical specimens. Reverse transcription and qPCR were then performed as described (*17*). Platinum SYBR Green qPCR SuperMix-UDG with ROX (Life Technologies) and ASB4-specific primers (forward: 5'-GCC TGC CAT TCT TGT CCT AA-3'; reverse: 5'-TGC ATG AGA GTC CTT GGA GA-3') was used for qPCR under the following condition: 50°C for 2 min for 1 cycle; 95°C for 10 min for 1 cycle; 95°C for 15 sec and 60°C for 1 min for 40 cycles. Expression level of ASB4 was normalized with the expression of ribosomal RNA 18S (for clinical specimens) or β-actin (for cultured cells). For obtaining the relative expression level of putative miRNAs against ASB4, qPCR was performed using each miRNA-specific primers (Qiagen, Hilden, Germany) for miR-200a, miR-200b, miR-383, miR-498, and miR-875 under the condition as stated in the manual: 95°C for 15 min for 1 cycle; 94°C for 15 sec, 55°C for 30 sec, and 70°C for 30 sec for 40 cycles.

### 2.4. *Ectopic expression and suppression of ASB4 in HCC cells*

Ectopic expression of ASB4 was achieved by transfecting ASB4-deficient Hep3B cells using Lipofectamine 2000 (Life Technologies) and human ASB4-expressing pcDNA3.1 vector (Life Technologies). Stable cell clones with suppressed expression of ASB4 were established in ASB4-expressing PLC and 97L cells by transfecting with Lipofectamine 2000 and pGFP-V-RS vector (OriGene Technologies, Rockville, MD, USA) harboring short hairpin RNA (shRNA). By screening with three shRNAs against exon 1 (GI326178/shRNA#1) or 2 (GI326179/shRNA#2 and GI326180/shRNA#3) of human ASB4, two shRNAs with the highest knockdown efficiency in each cell line were used for PLC and 97L cells.

### 2.5. *Cell proliferation assay*

Cultured HCC cells with manipulated expression of ASB4 were seeded on 96-well plates at 1,500 cells in each well. For each day after cell seeding for five days, 10 μL of MTT reagent was added to each well. The plate was then incubated at 37°C for 1.5 h before addition of 100 μL DMSO after removal of medium. Plates were then read to assess the cell proliferation rate of each group as before (*9,18*).

### 2.6. *Wound healing assay*

Cultured HCC cells with overexpression and knockdown of ASB4 were seeded on 6-well plates until cells reached about 90% confluency. A scratch was then created in each assayed well as described (*9,19*). Extent of cell migration towards the created wound was followed on every 12 h till 36 h.

2.7. *Cell invasion assay*

HCC cells ($8 \times 10^4$) with suppression of ASB4 were seeded in each BD BioCoat Matrigel Invasion Chamber (BD Biosciences, Bedford, MA, USA). To allow cell invasion through the chamber membrane, assayed cells resuspended in 0.5% FBS were placed within the chamber while medium with 2% FBS was added to the outer tank. After incubation for 48 h, 0.4% paraformaldehyde was used to fix the cells for 20 min before cells were stained with 1% crystal violet as before (*17*).

2.8. *Prediction of miRNA targeting ASB4*

Based on seed-matched sequence, miRWalk (*http://www.umm.uni-heidelberg.de/apps/zmf/mirwalk/*) (*20*) was used to predict potential miRNA with binding site on the 3'-untranslated region (3'UTR) of ASB4 and a sum score was used to rank each miRNA. In parallel, we also pulled out miRNA with expression negatively correlated with those of ASB4 based on our published miRNA expression array database of 97 HCC cases (*21*).

2.9. *Luciferase reporter assay to examine the binding of miR-200a to 3'UTR of ASB4*

3'UTR of ASB4 was obtained from 97L cells using primer sequences flanking the region of interest (forward: 5'-GCC TTA TGA GAC AGC AGT TCC CAA T-3' and reverse: 5'-GAA AGG AGA CAG AAG AGC TTT ATT T-3') before cloning into pMIR-REPORT Luciferase vector (Life Technologies), while pRL-TK vector (Promega, Madison, WI, USA) was used as background control. In addition to transfecting with these two vectors, H2M HCC cells were co-transfected with miR-200a mimic (Qiagen) and/or miR-200a inhibitor (Qiagen). Lipofectamine 2000 was used as transfecting agent. After 48 h, cells were harvested and suspended in Reporter Lysis Buffer (Promega) and subjected to dual luciferase reporter assay as before (*18,19*).

2.10. *Assessment of ASB4 expression upon ectopic expression of miR-200a*

Cellular miR-200a level was induced by transfecting 97L HCC cells using miR-200a mimic using Lipofectamine 2000. To support for the specificity of this experiment, exogenously induced miR-200a level was neutralized using miR-200a inhibitor as above in parallel experiments. The resultant effect of miR-200a mimic and inhibitor on ASB4 expression was assessed using qPCR as above.

2.11. *Statistical analyses*

Statistical analyses were performed using SPSS version 16 (SPSS Inc., Chicago, IL, USA) as described (*9*). Independent *t*-test was used to compare between experimental groups. Paired *t*-test was performed for comparing expression between tumors and adjacent non-tumor tissues. Clinicopathological features including HBsAg status, serum alpha-fetoprotein (AFP) level, presence of venous infiltration, tumor size, tumor differentiation and tumor stage were analyzed using Pearson's chi-squared test. A $p$-value $< 0.05$ was considered as statistical significance.

**3. Results**

3.1. *High expression of ASB4 in HCC tumors and its correlation with low serum AFP level*

Involvement of ASB4 in HCC was revealed by analyzing its expression in HCC clinical specimens. Retrospective analysis of published gene expression array database (*22-24*) associated high expression of ASB4 with tumor tissues when compared to the non-tumor tissues in 217 HCC cases ($p = 0.0013$) (Figure 1A, upper panel). Validation of the gene expression array data was achieved by detecting the level of ASB4 in a separate cohort of 50 cases using qPCR. A significant up-regulation of ASB4 was observed in HCC tumors when compared to their adjacent non-tumor counterparts ($p = 0.011$) (Figure 1A, lower panel). Among these 50 pairs of tissues, 27 cases with tumors having more than 2-fold induction in ASB4 expression, accounting for 54% of total studied cases. Clinical correlation was undertaken for these two cohorts of subjects separately. For each cohort, patients were further segregated into two groups based on expression of ASB4 in tumors, high expression of ASB4 denoted the top 20% of cases with highest expression. ASB4 expression did not correlate well with most of the studied clinical parameters including HBsAg status, tumor size, presence of venous infiltration, tumor differentiation and tumor stage. Unexpectedly, ASB4 expression was markedly correlated with serum AFP level both in the gene expression array cohort (Table 1a) and qPCR validation cohort (Table 1b), such that high expression of ASB4 linked to low serum AFP level condition.

3.2. *Role of ASB4 in conferring migration and invasion properties of HCC cells*

To provide further evidence to support the tumorigenic properties of ASB4 in HCC, next we examined the phenotypic effects of HCC cells when ASB4 level was

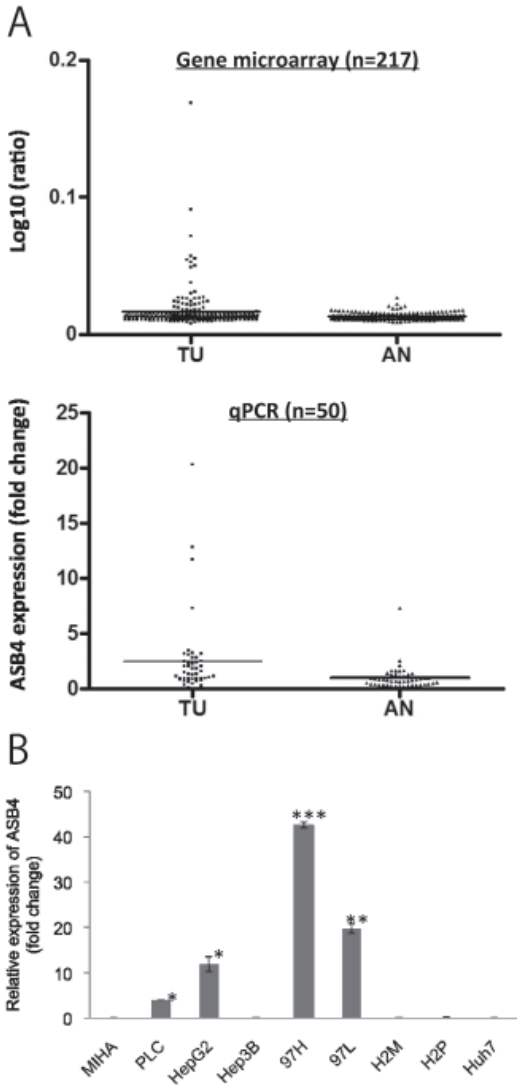


**Figure 1. High expression of ASB4 in HCC tumors and cultured cell lines. (A)** Gene expression array of 217 HCC cases was re-analyzed for the expression of ASB4 in tumors and adjacent non-tumor tissues. Validation was further performed in a separate cohort of 50 cases using qPCR. Both assays show significant up-regulation of ASB4 in tumors when compared to the non-tumor tissues. For gene expression array, log10 (ratio) of ASB4 in each sample was obtained by subtracting with the mean log10 (intensity) of ASB4 across all non-tumor tissues ($p = 0.0013$). For qPCR, the expression of ASB4 was obtained by normalization with ribosomal RNA 18S. The fold change was then determined by normalizing with the average value of non-tumor tissues ($p = 0.011$). **(B)** Differential expression of ASB4 was observed in our human cell line panel using qPCR. Among the eight HCC cell lines, four cell lines, PLC, HepG2, 97H and 97L, were found to have high expression of ASB4. The expression levels were normalized to average of the cell line panel. Representative set among 4 sets of experiments is shown. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. AN, adjacent non-tumor tissues; TU, tumor.

manipulated. For this purpose, ASB4 was suppressed in two HCC cells having high expression of ASB4, PLC and 97L (Figure 1B), by treating them with shRNA to generate stable clones with suppressed level of ASB4. When assessed using qPCR, individual shRNA exerted different degrees of ASB4-suppressing efficiencies in different cell lines, such that shRNA#2 led to the most

**Table 1a. Clinical correlation of ASB4 in 217 HCC cases***

| Clinicopathological features | Patient number | ASB4 expression | | *p*-value |
|---|---|---|---|---|
| | | Low | High | |
| HBsAg | | | | 0.142 |
| Negative | 30 | 21 | 9 | |
| Positive | 187 | 153 | 34 | |
| Serum AFP level (ng/mL) | | | | 0.003 |
| < 50 ng/mL | 97 | 69 | 28 | |
| ≥ 50 ng/mL | 120 | 105 | 15 | |
| Serum AFP level (ng/mL) | | | | 0.011 |
| < 100 ng/mL | 108 | 79 | 29 | |
| ≥ 100 ng/mL | 109 | 95 | 14 | |
| Tumor size (cm) | | | | 0.466 |
| < 5 cm | 66 | 51 | 15 | |
| ≥ 5 cm | 151 | 123 | 28 | |
| Venous infiltration | | | | 0.865 |
| Absent | 107 | 85 | 22 | |
| Present | 110 | 89 | 21 | |
| Tumor differentiation[a,b] | | | | 0.53 |
| Well | 37 | 26 | 11 | |
| Moderately | 114 | 92 | 22 | |
| Poorly | 38 | 31 | 7 | |
| Undifferentiated | 2 | 2 | 0 | |
| Tumor stage[c] | | | | 0.893 |
| Stage I | 92 | 72 | 20 | |
| Stage II | 64 | 51 | 13 | |
| Stage IIIA | 45 | 37 | 8 | |
| Stage IIIB | 14 | 12 | 2 | |
| Stage IV | 2 | 2 | 0 | |

*, Analysis of gene expression array database; [a], Calculation was performed based on available data; [b], Determined based on Edmonson grading; [c], Tumor was staged using new AJCC system.

**Table 1b. Clinical correlation of ASB4 in 50 HCC cases***

| Clinicopathological features | Patient number | ASB4 expression | | *p*-value |
|---|---|---|---|---|
| | | Low | High | |
| HBsAg[a] | | | | 0.8 |
| Negative | 7 | 6 | 1 | |
| Positive | 42 | 33 | 9 | |
| Serum AFP level (ng/mL) | | | | 0.011 |
| < 50 ng/mL | 21 | 13 | 8 | |
| ≥ 50 ng/mL | 29 | 27 | 2 | |
| Serum AFP level (ng/mL) | | | | 0.035 |
| < 100 ng/mL | 24 | 16 | 8 | |
| ≥ 100 ng/mL | 26 | 24 | 2 | |
| Tumor size (cm) | | | | 0.258 |
| < 5 cm | 13 | 12 | 1 | |
| ≥ 5 cm | 37 | 28 | 9 | |
| Venous infiltration | | | | 0.707 |
| Absent | 16 | 12 | 4 | |
| Present | 34 | 28 | 6 | |
| Tumor differentiation[a,b] | | | | 0.177 |
| Well | 6 | 3 | 3 | |
| Moderately | 28 | 22 | 6 | |
| Poorly | 9 | 8 | 1 | |
| Undifferentiated | 0 | 0 | 0 | |
| Tumor stage[c] | | | | 0.189 |
| Stage I | 9 | 7 | 2 | |
| Stage II | 16 | 15 | 1 | |
| Stage IIIA | 12 | 10 | 2 | |
| Stage IIIB | 13 | 8 | 5 | |
| Stage IV | 0 | 0 | 0 | |

*, Based on qPCR data generated in this study; [a], Analyses were performed based on available data; [b], Tumor differentiation was determined based on Edmonson grading; [c], Tumor was staged using new AJCC system.

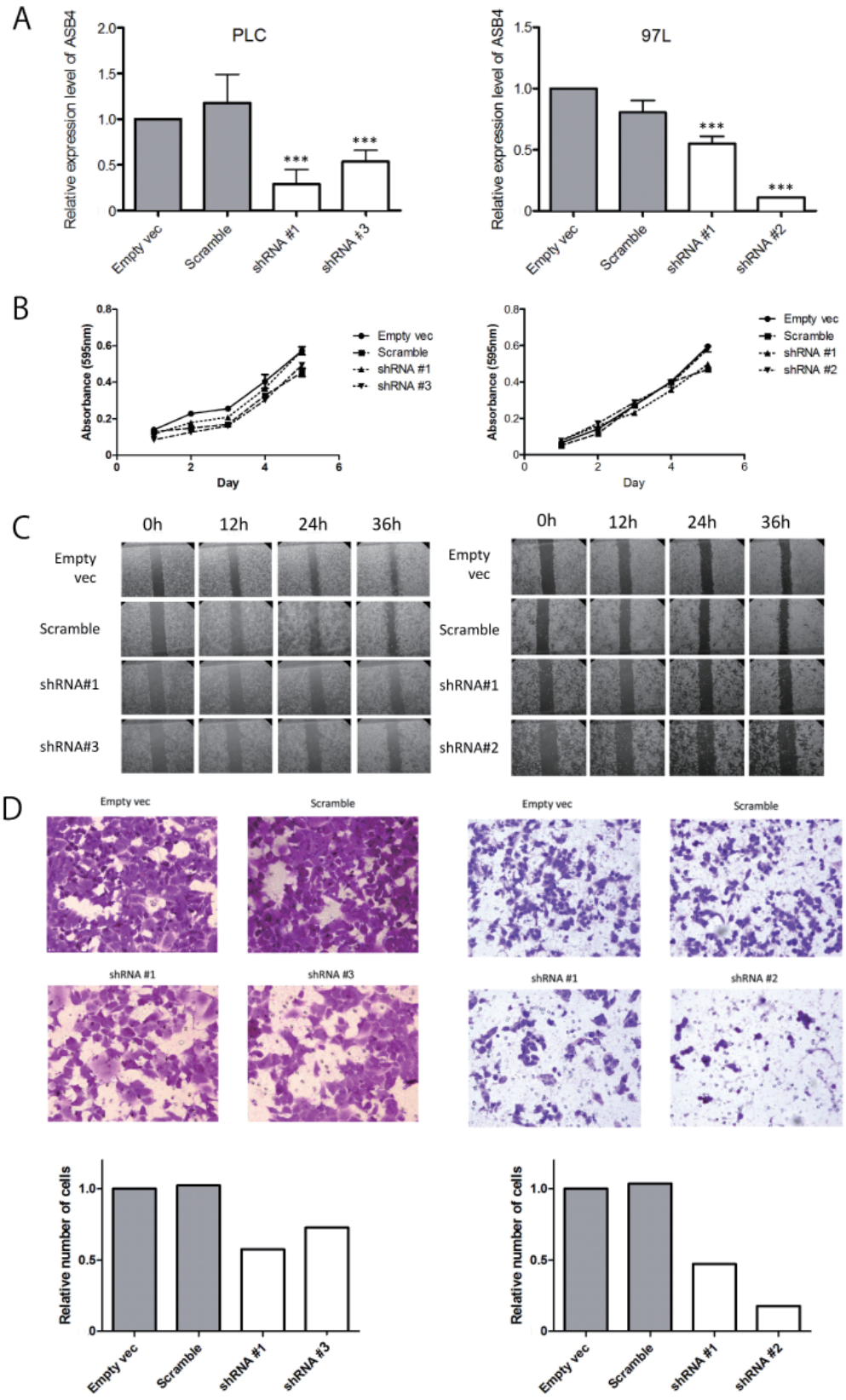


**Figure 2. Suppression of ASB4 inhibits migration and invasion of PLC and 97L cells. (A)** PLC and 97L cells were transfected with ASB4-targeting shRNAs to develop stable cell clones with suppression of ASB4. In parallel, cells were transfected with scrambled shRNA (Scramble) and empty vector (Empty vec) as negative controls. Expression of ASB4 in Scramble and shRNA was normalized against the expression in Empty vec. **(B)** Using MTT assay, no significant difference in proliferation rate was observed when ASB4 was suppressed in PLC and 97L cells. **(C)** After suppression of ASB4, shRNA clones adopted slower migration rate towards the artificially created wounds when compared to Empty vec and Scramble cells. Photos of the same positions of each were taken at three time points (12 h, 24 h and 36 h) after scratching on the cell monolayer. **(D)**. 48 h after seeding cells on the inner chamber, invaded cells from each experiment group were stained and counted. Less number of invaded cells was recorded for shRNA clones when compared to Empty vec and Scramble groups. Original magnification, 200×. The number of invaded cells in each group was counted and normalized to Empty vec in the bar chart. ***, $p < 0.001$.

prominent suppression of ASB4 in 97L rather than others (Figure 2A). Using MTT assay, we failed to observe any significant deviation in proliferation rates of ASB4-suppressed PLC and 97L cells when compared to their parallel controls transfected with empty vector or scrambled shRNA (Figure 2B). Intriguingly, suppressing ASB4 in PLC and 97L cells retarded the migration of these cells towards artificially created wounds in confluent cell monolayers. ASB4 seems to determine the migration ability of HCC cells, as shRNA#2-transfected cells with most significant knockdown in ASB4 level were incapable to close the wound even after 36 hours of incubation (Figure 2C). Besides cell migration, ASB4 is also responsible for regulating the invasive properties of HCC cells. For those PLC and 97L cells with suppressed level of ASB4, fewer number of cells successfully invaded through the Matrigel-coated membrane of the invasion chamber. Such drop in numbers also corresponded to the extent of ASB4 suppression. Similar to the observation of wound-healing assay, transfection of shRNA#2 into 97L cells resulted in the least number of cells with penetrative properties (Figure 2D).

Using opposite approach against the knockdown experiment, ectopic expression of ASB4 was performed in ASB4-deficient Hep3B cells by transient transfection of ASB4-expressing vector (Figure 3A). As in Figure 2B, MTT assay was unable to detect significant changes in the proliferation of ASB4-expressing Hep3B cells in comparison to those cells transfected with empty vector (Figure 3B). Migration rate of Hep3B cells enhanced dramatically after transfection of ASB4-expressing vector, as the artificially created wound was nearly closed after 36 hours (Figure 3C). Based on this set of knockdown and overexpression experiment, it is conclusive to know for the role of ASB4 in conferring migration and invasion properties of HCC cells, despite the involvement of ASB4 in cell proliferation is rather limited.

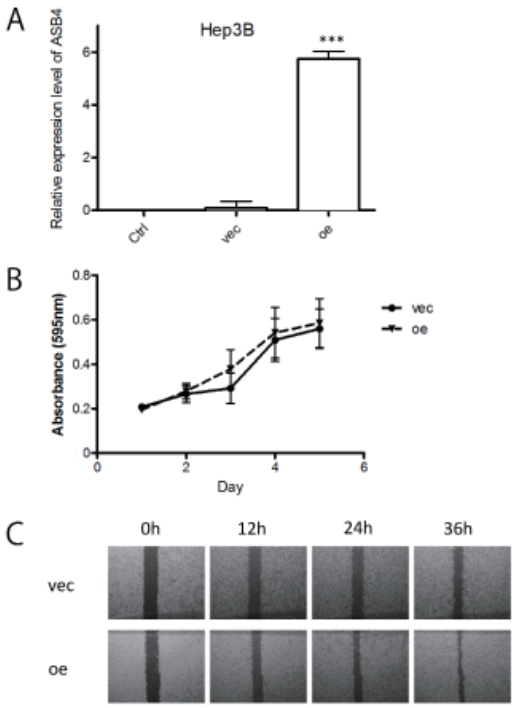


**Figure 3. Overexpression of ASB4 promotes migration of Hep3B cells. (A)** Transient transfection of ASB4 was performed in Hep3B cells. By means of qPCR, a significant induction in the level of ASB4 was observed in ASB4-overexpressing cells (oe) when compared to no treatment control (Ctrl) and cells transfected using empty vector (vec). ASB4 expression in oe and vec was normalized to Ctrl. **(B)** No significant difference in cell proliferation was observed between oe and vec group with or without overexpression of ASB4 when assessed using MTT assay. **(C)** Wound-healing assay was performed using vec and oe cells, in which overexpression of ASB4 enhanced the migration of cells towards the artificially created wound. Photos of the same positions of each were taken 12 h, 24 h and 36 h after scratching of the cell monolayer. ***, $p < 0.001$.

### 3.3. *Regulation of ASB4 by miR-200a*

MiRNA belongs to a class of small molecule capable of regulating dozens of cancer-related molecules *via* its binding to the 3'UTR of target molecules (*25,26*). Based on the above findings pointing to the role of ASB4 in HCC, we hypothesize ASB4 might also be subjected to this kind of post-translational regulation. To prove for this notion, potential miRNAs binding to the 3'UTR of ASB4 were predicted bioinformatically. Table 2 shows 11 miRNAs carrying seed-matched sequences for 3'UTR of ASB4. Taking advantage of our miRNA database of 97 HCC cases with matched gene expression profile (*21-24*) and by performing correlation analysis using ASB4 expression level, 3 miRNAs negatively correlated with ASB4 expression were identified on top of the list (Table 3). Cross comparison of miRNAs in Table 2 and 3 pointed to miR-200 family members (miR-200a and miR-200b) as potential miRNAs regulating ASB4 in HCC. These two miRNAs together with those miRNAs (miR-383, miR-498, and miR-875) absent from our miRNA expression database (*21*) were subjected to qPCR to detect their relative expressions in our cell line panel as in Figure 1B (Figure 4A). Amongst these five miRNAs, miR-200a, miR-200b, and miR-383 displayed a general, yet not perfect, trend for its expression negatively correlated to ASB4 level in this cell line panel. Of note, some cell lines having negligible levels of both studied miRNA and ASB4 were not taken into account for consideration. Applying these selection criteria has then put our focus on miR-200a for validation study.

In understanding whether miR-200a could interact with 3'-UTR of ASB4, H2M cells lacking both miR-200a and ASB4 were transfected with vector harboring 3'UTR of ASB4. Co-transfection with miR-200a mimic reduced the luciferase activity. This reduction was further enhanced when a higher amount of miR-200a mimic was used. On the contrary, the reduction in luciferase activity was rescued when miR-200a inhibitor was co-transfected with mimic (Figure 4B). This data has demonstrated exogenous addition of miR-200a can bind specifically to

**Table 2. miRNAs with potential binding sites on 3'UTR of ASB4**

| MicroRNA | StemLoop ID | DIANAmT | miRanda | miRDB | miRWalk | RNAhybrid | PICTAR4 | PICTAR5 | PITA | RNA22 | Targetscan | SUM |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| hsa-miR-498 | hsa-mir-498 | 1 | 1 | 0 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 7 |
| hsa-miR-143 | hsa-mir-143 | 1 | 1 | 0 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 7 |
| hsa-miR-200a | hsa-mir-200a | 1 | 1 | 0 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 6 |
| hsa-miR-875-3p | hsa-mir-875 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 0 | 1 | 6 |
| hsa-miR-148b | hsa-mir-148b | 1 | 1 | 0 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 6 |
| hsa-miR-648 | hsa-mir-648 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 0 | 1 | 6 |
| hsa-let-7f | hsa-let-7f-2 | 1 | 1 | 0 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 6 |
| hsa-miR-218 | hsa-mir-218-2 | 1 | 1 | 0 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 6 |
| hsa-let-7a | hsa-let-7a-3 | 1 | 1 | 0 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 6 |
| hsa-miR-331-5p | hsa-mir-331 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 0 | 1 | 6 |
| hsa-miR-383 | hsa-mir-383 | 1 | 1 | 0 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 6 |

**Table 3. miRNAs with expression negatively correlated with those of ASB4 in 97 HCC cases**

| miRNA | Spearman R | *p*-value (R) | ASB4 (T) | ASB4 (NT) | ASB4 (NT/T) | *p*-value (T versus NT) |
|---|---|---|---|---|---|---|
| HSA-MIR-138 | -0.314 | 0.002 | 0.022638 | 0.039637 | 1.750929 | 0.000676 |
| HSA-MIR-200B | -0.334 | 0.001 | 0.990351 | 1.505138 | 1.519802 | 0.027175 |
| HSA-MIR-375 | -0.242 | 0.020 | 1.063168 | 1.653897 | 1.555631 | 0.023164 |

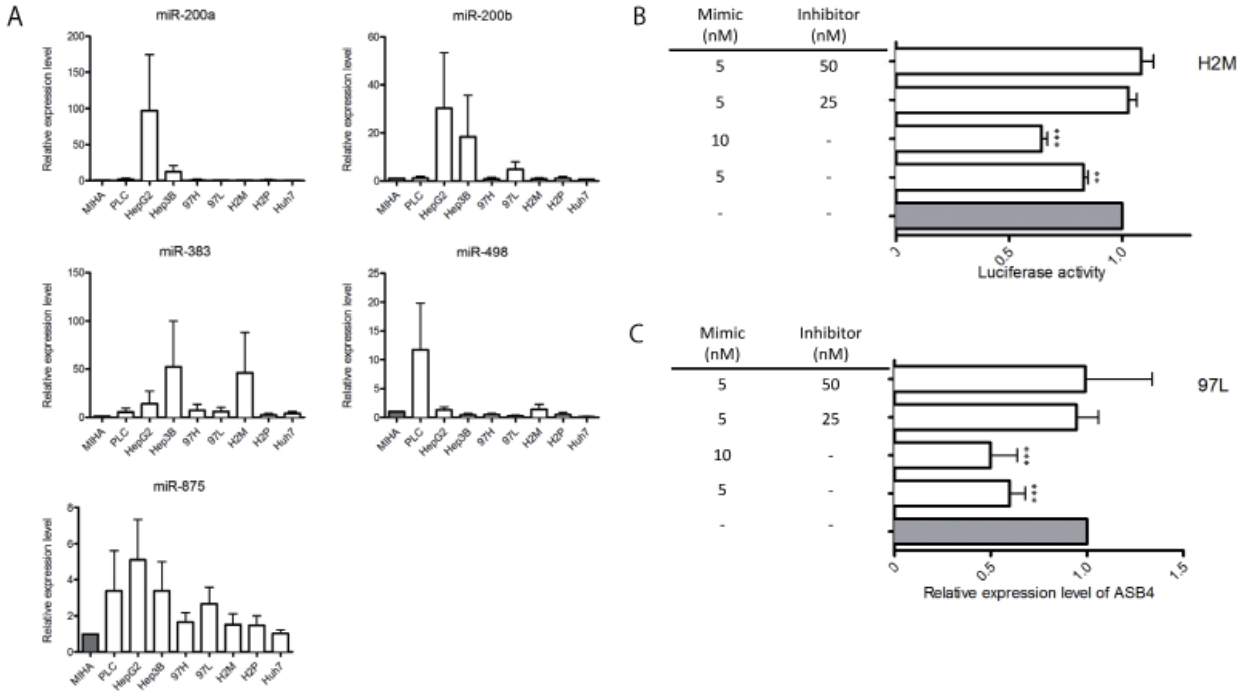


**Figure 4. MiR-200a binds and suppresses the level of ASB4. (A)** Five miRNAs chosen based on the selection criteria mentioned in the result section were subjected to qPCR to determine their expression in our panel of liver cell line. Differential expression of each miRNA was observed. For each cell line, expression of each miRNA in HCC cells was normalized against immortalized normal hepatocyte MIHA. **(B)** Luciferase reporter assay was performed to investigate whether miR-200a would bind to 3'UTR of ASB4 in H2M cells lacking both of these molecules. Transfection of H2M cells with miR-200a mimic and 3'UTR of ASB4 reduced luciferase activity in a dose-dependent manner, while addition of miR-200a inhibitor restored the drop in activities. Luciferase activity of each treatment was normalized to control cells transfected with 3'UTR of ASB4. **(C)** After transfecting ASB4-expressing 97L cells with miR-200a mimic, ASB4 suppression was measured. The specificity of this reaction was confirmed when this ASB4-suppressing effect was neutralized with the addition of miR-200a inhibitor. The relative expression of each treatment group was normalized to control cells without any treatment. **, $p < 0.01$; ***, $p < 0.001$.

3'UTR of ASB4.

Following the luciferase reporter assay, plan has been devoted to examine whether miR-200a mimic can reduce the endogenous level of ASB4. For this, 97L cells were used due to its absence of miR-200a and its expression of ASB4. By means of qPCR, dose-dependent reduction in ASB4 level was observed in 97L cells after transfection with miR-200a mimic. The specificity of this reaction was confirmed as co-transfecting cells with both miR-200a mimic and inhibitor ruled out the ASB4-reducing effects of miR-200a (Figure 4C). Together with the luciferase reporter assay result, miR-200a can bind to 3'UTR of ASB4 and reduce its level.

## 4. Discussion

Involvement of ASB4 in HCC was first hinted in our previous proteomic study of developing mouse livers (*10*). Expression of ASB4 was detected in fetal mouse livers, but was absent in adult mouse livers. In the same study, ASB4 was found to have negligible expression in an immortalized normal hepatocyte line MIHA and differential expression was noticed in several HCC cell lines. The high expression of ASB4 in metastatic 97L and 97H cell lines has prompted us to include more cell lines in this study, among the three additional cell lines one of them H2M is a metastatic line. Although we would expect H2M cells also possess high level of ASB4, no measurable level of ASB4 was detected in this pair of cell line (primary H2P and metastatic H2M). Only PLC cells had detectable level of ASB4. The differential expression of ASB4 in our cell line panel has implicated that a subset of HCCs requires specifically the function of this molecule to maintain its tumor properties. To follow on this statement, ASB4 level was measured in clinical specimens of HCC. Our earlier investigation failed to detect ASB4 under the stated experimental condition (*10*), for which this problem was remedied by using another set of qPCR primer in this study. The primer used in our previous setting can detect both variants 1 and 2 of ASB4, while the current primer can only recognize variant 1. 54% of patients are found to have ASB4-overexpressing tumors. However, high expression of ASB4 does not correlate with most of the examined clinicopathological parameters including presence of venous infiltration and tumor stages. Despite that, high expression of ASB4 was found associating with low serum level of AFP. Elevated AFP level has been used as a serological marker for HCC detection and used in most clinical centers, together with ultrasound, around the world for routine surveillance of HCC (*27,28*). Nevertheless, using AFP as a marker for HCC fails to cover all HCC cases because around 20-50% of cases have comparable AFP level as healthy individuals (*29-31*). Continuing efforts have identified supplemental markers for AFP in detecting AFP-negative subjects. Prominent molecule like dickkopf-1 is proven to supersede or complement AFP for this purpose (*32*). Apart diagnosis, AFP also has clinical value in classifying poor prognostic cases such as those with large tumor size and with portal vein thrombus (*29,30*). Its level negatively correlated with those of AFP, ASB4 might be another attractive candidate for HCC prognostication.

Embryogenesis and tumorigenesis are distinct cellular processes. Yet, they share certain common pathways and possess a pool of oncofetal molecules. The first and most well-known example of liver oncofetal molecule is AFP (*33*). Its clinical applicability in HCC surveillance is well recognized (*34*). Despite that, measurement of serum AFP suffers from its own limitation in sensitivity and specificity. To improve this clinical situation, other oncofetal molecules soluble in circulation like glypican-3 and GP73 have been tested for their potential use, or in combination with serum AFP, in detecting HCC (*35-37*). Adding value to the clinical usefulness of oncofetal molecules, some of them have also been examined for their therapeutic potentials. Representative candidates include cadherin-17 and survivin, for which suppression of their levels is known as a way to counteract liver tumorigenesis in both *in vitro* and *in vivo* experimental settings (*17,38*). In this study, an alleviation of migration and invasion in HCC cells was clearly shown upon ASB4 suppression, providing a solid evidence for the involvement of ASB4 in the presentation of these tumor phenotypes. Interestingly, we did not observe any variation in proliferation of HCC cells after ASB4 suppression. This undoubtedly supports for the selective involvement of ASB4 in cell migration and invasion.

ASB4 is shown to express in a subset of HCC based on the qPCR data derived from cultured cells and clinical specimens. Next, we might want to understand what factor leads to its upregulation in ASB4-expressing HCC. MiRNAs execute their biological functions *via* binding on the 3'UTR of target molecules, thereby interfering target gene expressions under normal and diseased conditions (*39*). A growing number of reports have further complicated the current network of miRNA in HCC (*40,41*). MiRNAs participate in tumorigenesis by modulating the balance between oncogene and tumor suppressor gene (*42,43*). Attempt has been made to investigate whether ASB4, an oncofetal molecule, is subjected to miRNA-based regulation. Results derived from this study have confirmed the ASB4-inhibiting effect of miR-200a. Indeed, down-regulation of miR-200a is observed in HCC and that suppression of miR-200a promotes tumor phenotypes of HCC cells (*44*). Notably, overexpression of miR-200a in HCC cells did not alter cell proliferation, but is able to decrease cell migration by increasing E-cadherin (*45*). These earlier studies are in support for our observations showing exogenous addition of miR-200a reduces ASB4 level. We have also indicated that suppression of ASB4 alleviates migration and invasion, but not proliferation, of HCC cells, for which this result highly resembles to those exhibited by overexpressing miR-200a. This is the first study that deciphers miRNA-based regulatory mechanism of ASB4. Apart from this mode of regulation, there is only one study so far describing ASB4 as a downstream molecule of NF-κB and TNF-α signaling pathway in endothelial cells (*15*). In conclusion, the current study confirms the tumorigenic properties of ASB4 and introduces a novel regulatory mechanism for this molecule in HCC.

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