Prognostic and Functional Role of Hyaluronan-binding Protein 1 (HABP1) in Pancreatic Ductal Adenocarcinoma

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Abstract

Background: Hyaluronan-binding protein 1 (HABP1) is one of molecules that binds to hyaluronan and is involved in a variety of cellular processes including cell proliferation and migration. HABP1 has related to the progression of various cancers however there are few reports on the expression and function of HABP1 in pancreatic ductal adenocarcinoma (PDAC). We examined the expression and functional role of HABP1 in PDAC.

Methods: (1) Immunohistochemical analysis of HABP1 protein was done in archival tissues from 105 PDAC patients. (2) We examined the functional effect of HABP1 on proliferation, colony formation, and migration in PDAC cells by knockdown of HABP1.

Results: (1) HABP1 was overexpressed in 49(46.2%) of 106 PDAC patients. Overall survival was significantly shorter in patients with high HABP1 expression (median survival time of 12.8 months) than in those with low HABP1 expression (28.5 months) (log-rank test; p = 0.004). (2) Knockdown of HABP1 expression in PDAC cells resulted in decreased cell proliferation, colony formation and migration ability.

Conclusion: HABP1 may serve as a prognosis factor in PDAC and could be a new therapeutic target.

1. Background

Pancreatic ductal adenocarcinoma (PDAC) is one of the most aggressive neoplasms ranking the fourth among cancer-related deaths in western countries [1]. Currently, multidisciplinary treatments such as surgery and chemoradiotherapy are used to treat pancreatic cancer, but the survival rate has not been dramatically improved. In addition, only a limited number of patients with PDAC may benefit from new treatment modalities including immune checkpoint inhibitors and precision medicine based on genome-wide molecular alterations. Therefore, it is necessary to approach novel therapeutic strategies based on comprehension of the biological and molecular mechanisms fundamental the offensive progression of PDAC.

Recently, the focus of cancer research has shifted to surrounding microenvironment of cancer cells. PDAC character has been a dense stroma comprising of various stromal cells and rich extracellular matrixes (ECM) [2]. Hyaluronan (HA), a major component of ECM, is exuberantly accumulated in a surrounding microenvironment of various cancers including PDAC and acts an important role in a variety of cellular processes including cell invasion, migration, proliferation, and etc.[3−9]. In addition, low-molecular-weight HA (LMW-HA) or has been reported to be more indispensable for cancer progression in terms of invasion and metastasis rather than high-molecular weight HA (HMW-HA)[10−13]. We also provided that LMA-HA accumulation correlates with motility PDAC cells[3]. HA, a large linear glycosaminoglycan of up to 106−107 Da in its naïve form, is produced by HAS enzymes (HASs) and degraded into smaller fragments by hyaluronidases (HYALs). We already reported high expression of HAS2 (one of the HAS proteins) in PDAC was related with poor survival after surgery[14]. In addition to the HA synthesis, division from large HA to small HA fragments by HYALs or other enzymes (including

KIAA1199) is also accelerated in malignant tumors. In our previous study, HYAL1, KIAA1199 (CEMIP) have been shown to be overexpressed in PDAC [15].

In this study, we focused on hyaluronan-binding protein 1 (HABP1), one of the hyaluronan-binding proteins. HABP1 is initially called the globular head receptor for complement component 1q (gC1qR), and is known to be a protein that inhibits C1 activation. Abnormal expression and/or function of HABP1 are reported in neurodegenerative diseases, impaired glucose tolerance, and cancer [16–25]. Especially, HABP1 has an important role in cancer initiation and progression [26] [27]. There are few reports on the expression and role of HABP1 in PDAC [24]. Here, we investigated the expression, clinicopathological significance, and biological function of HABP1 in pancreatic cancer.

2. Methods

Immunohistochemistry (IHC)

This study used archival tissues obtained from 105 consecutive patients who underwent surgery at the Department of Surgery 1, School of Medicine, University of Occupational and Environmental Health. All of these specimens were properly used after obtaining patients' written informed consent, and this study was approved by the Ethics Committee of School of Medicine, University of Occupational and Environmental Health.

Each section was incubated in a 1:100 dilution of anti-HABP1 antibody (Monoclonal Anti-C1QBP antibody produced in mouse sc-23885 Sigma-Aldrich, St. Louis, MO) for an hour. The IHC reaction was quantified by multiplying staining intensity by the percentage of positive tumor cells. The staining intensity was graded as follows: 0 (no staining), 1 (weak staining), 2 (moderate staining), and 3 (strong staining). The percentage (0 to 100%) of the extent of reactivity was scored as follows: 0 (no positive tumor cells), 1 ($\leq 10\%$), 2 ($10–50\%$), and 3 ($\geq 50\%$) [28]. Each case was scored independently by two investigators in a blinded manner. Scores ≤ 4 were regarded as negative expression, and the remainder were classified as positive expression.

Cell culture and reagents

We used 2 PDAC cell lines, PANC-1 (American Type Culture Collection, Manassas, VA, USA), and NOR-P1 (RIKEN BRC Cell Bank, Tsukuba, Ibaraki, Japan) which are high expression of HABP1 in our possession. An immortalized cell line derived from human pancreatic duct, HPDE, was a kind gift from Dr. M.S. Tsao (Univ. of Toronto, Canada). PDAC cell lines were maintained in RPMI1640 medium (Life Technologies, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) (Life Technologies, Grand Island, NY, USA) and 1% streptomycin and penicillin (Life Technologies, Grand Island, NY, USA). HPDE was maintained in HuMedia-KG2 (KURABO, Kyutaro-machi, Chuo-ku, Osaka, Japan), in a 5% CO_2 incubator at 37 °C.

siRNA targeting for HABP1

The siRNA targeting for HABP1 (ON-TARGETplus Smart Pool Human HABP1 L-011225-01-0005) and negative control siRNA (ON-TARGETplus Control siRNA Non-Targeting siRNA #1 D-001810-01-05) were purchased from GE healthcare (Chicago, IL, USA). NOR-P1 and PANC-1 were transfected with 100 nM siRNA using DhermaFECT 1 Transfection Reagent (GE healthcare, Chicago, IL, USA) according to manufacturer's instructions. After 48 hours treatment, the cells were immediately used for further experiments.

Quantitative real-time RT-PCR

Total RNA was isolated from cell lines using RNeasy Mini Kit (QIAGEN GmbH, Hilden, Germany) according to the manufacture's protocol. First strand cDNA was synthesized from 1.0 µg of total RNA using SuperScript® VILO cDNA synthesis Kit and Master Mix (Thermo Fisher Scientific Inc., Waltham, MA, USA). Real-time mRNA expression analysis of HABP1 and a housekeeping gene (GAPDH) for control was performed using TaqMan@ Gene Expression Assays and Step One Plus real-time PCR system (Thermo Fisher Scientific Inc., Waltham, MA, USA) according to the manufacture's instruction. The assay numbers for these genes were as follows: Hs00241825_m1 (HABP1); and Hs02758991_g1 (GAPDH).

The relative quantification was given by the Ct values, determining the reactions for target genes and an internal control gene in all samples.

Cell Proliferation Assay

PDAC cells treated with siRNA targeting for HABP1 and negative control siRNA were incubated for 1, 3, and 5 days and counted using trypan blue staining.

Colony Formation Assay

After treatment with siRNA, PDAC cells were harvested, counted, and the same number of cells in each group were seeded in dishes. Cells were grown for 14 days and colonies were fixed and stained with hematoxylin and eosin. We counted the number of colonies on each dish.

Migration assay

The migratory ability of cells was determined by transwell cell migration assay using cell culture inserts equipped with a filter membrane containing 8 µm pores (BD Biosciences, Franklin Lakes NJ). The lower chamber was filled with RPMI1640 containing 10% FBS. The upper chamber was filled with 2.0×10^4 cells (for PANC-1) or 5.0×10^4 cells (for NOR-P1) in the RPMI1640. After 24 h incubation, the cells remaining on the upper side of the filters were removed. The cells on the bottom surface of the membrane were stained with hematoxylin and eosin and the number of cells that had migrated to the bottom surface of the membrane were counted in five randomly selected microscopic fields in each sample.

Western blot analysis

The cells were harvested and total protein was extracted with PRO-PREP protein extraction solution (iNtRON Biotechnology Inc., Seongnam-si, Gyeonggi-do, Republic of Korea). Equal amount of protein was subjected to 12% Mini-PROTEAN Precast Gel (Bio-Rad Laboratories, Inc., Philadelphia, PA, USA) and transferred on to PVDF membranes (ATTO, Daito-ku, Tokyo, JAPAN). Membranes were blocked for 1 h with 3% BSA (Sigma-Aldrich, St. Louis, MO, USA) in TBST buffer at room temperature, then were incubated with antibodies against HABP1 (Monoclonal Anti-C1QBP antibody produced in mouse sc-23885, Sigma-Aldrich, St. Louis, MO, USA) and ß-Actin (Proteintech Group, Rosemont, IL, USA) for overnight at 4 °C, followed by incubation with secondary antibodies (Proteintech Group, Rosemont, IL, USA) for 1 h at room temperature. The proteins were visualized using an ECL Western Blotting Detection System (GE healthcare, Chicago, IL, USA).

Statistical analysis

Statistical analyses were performed using SPSS statistical software version 25.0 (SPSS, Chicago, Illinois, USA). Chi-square tests, Student's t test and Mann-Whitney U test were used for group comparison. The Kaplan–Meier survival curves and log rank tests were used for survival analysis. Prognostic factors were evaluated by univariate and multivariate analyses using Cox proportional hazard regression models. A p-value of < 0.05 was considered statistically significant.

3. Results

Immunohistochemical analysis and prognostic relevance of HABP1 in PDAC

We used immunohistochemical analysis to determine expression patten of HABP1 in PDAC. HABP1 expression was negative or only slightly positive in normal pancreata including ductal cells, acinar cells, and islet cells, whereas HABP1 was highly expressed in some tumor cells. Staining was detected in membrane and/or cytoplasm of the tumor cells (Fig. 1).

Of 105 PDAC cases, 49 (46.7%) showed high HABP1 expression, whereas the remaining 56 (53.3%) showed low expression, according to our staining quantification criteria. Clinicopathological data were compared between high HABP1 expression group and low HABP1 expression group (Table 1). Tumor size was significantly larger in high HABP1 expression group than in low HABP1 expression group (3.2 (0.6-8) cm vs 2.7 (0.6-7) cm, p = 0.00883). There was no difference between the groups in other clinicopathological variables including age, gender, tumor location, levels of tumor makers, UICC stages, and other pathological factors.

We then compared survival between the groups. The overall survival was significantly shorter in patients with high HABP1 expression (median survival time of 12.8 months) than in patients with low HABP1 expression (median survival time of 28.5 months) (log-rank test; p = 0.004) (Fig. 2, Kaplan-Meier survival curve).

Prognostic factors were examined using Cox proportional hazard regression models. Univariate analysis revealed high HABP1 expression ($p < 0.001$), preoperative high CA19-9 levels ($p = 0.031$), and large tumor size ($p = 0.005$), to be significantly associated with poor prognosis (Table 2). Multivariate analysis showed each clinicopathological factors mentioned above was an independent prognostic factor (Table 2).

Functional analysis of HABP1 in PDAC cell line

First, we investigated mRNA expression of HABP1 in a panel of 10 PDAC cell lines. HABP1 mRNA was highly expressed in 5 (50%) of 10 PDAC cell lines (as compared to a control cell line HPDE) (Fig. 3A). Of these cell lines, two cell lines (NOR-P1 and PANC-1) with high HABP1 expression were then used for further experiments.

We used siRNA to knockdown the expression of HABP1 in NOR-P1 and PANC-1 with high HABP1 mRNA expression. Real-time RT-PCR showed that transfection with siRNA targeting for HABP1 (siRNA HABP1) resulted in 97%-99% knockdown in these cell lines (Fig. 3B and 3C). Western blot analysis validated the successful knockdown of HABP1 expression at protein level (Fig. 3D).

We examined proliferation, colony formation, and migration in NOR-P1 and PANC-1 cells with or without knockdown of HABP1 to reveal biological function of HABP1 in pancreatic cancer. First, we examined whether HABP1 knockdown affected PDAC cell proliferation. Cell counting assay showed the knockdown of HABP1 significantly decreased the proliferation of PDAC cells as compared to control on day3 and day 5 (NOR-P1: $p < 0.001$, PANC-1: $p = 0.041$) (Fig. 4A). Next, the number of colonies was significantly lower in HABP1 knockdown cells than in control cells in both cell lines (NOR-P1: $p = 0.011$, PANC-1: $p = 0.038$) (Fig. 4B). Finally, we investigated whether HABP1 affected cell migration. The transwell migration assay showed that knockdown of HABP1 significantly inhibited the migration of PDAC cells as compared to control the migratory ability in both cell line (NOR-P1: $p < 0.001$, PANC-1: $p < 0.001$) (Fig. 4C).

4. Discussion

In the present study, we investigated expression and functional significance of HABP1 in PDAC. The major findings obtained were as follows. (1) HABP1 was highly expressed in 46% of patients with PDAC. (2) The survival of PDAC patients with high expression of HABP1 was significantly shorter than those with low expression of HABP1. (3) Multivariate analysis identified high HABP1 expression as an independent factor predicting poor prognosis. (4) Knockdown of HABP1 in PDAC cells resulted in decreased proliferation, colony formation, and migratory ability. These finding suggest that HABP1 may play a role in aggressive phenotype of PDAC.

HABP1 is a multi-functional glycoprotein ubiquitously expressed in various tissues. It is involved in a variety of cellular processes, including cell motility, senescence, apoptosis, and autophagy [23]. Interestingly, elevation of HABP1 induces chronic generation of reactive oxygen species (ROS) [29], and the generation of excess ROS supports the survival of cancer cells by contributing to extra energy

production for growth [30]. Recently, it has been shown that HABP1 overexpression triggers induction of senescence in fibroblasts [31]. These functions of HABP1 support its important role in cancer initiation and progression. In fact, overexpression of HABP1 in HepG2 cells leads to enhanced cell survival and tumorigenicity by activating HA-mediated cell survival pathways [26] [27]. Similarly, exogenous administration of HABP1 protein enhances migration and tumor growth of melanoma [32]. The functional relevance of HABP1 in PDAC remains unknown. We demonstrate, for the first time, that siRNA knockdown of HABP1 resulted in decreased proliferation, colony formation, and migration of PDAC cells. These findings suggest that HABP1 expression is involved in progression of PDAC, as well as other cancer types.

We demonstrate that high HABP1 expression is associated with shorter survival in patients with PDAC who underwent surgery. Consistent with our present results, it was recently reported that high cytoplasm (but not nucleus) HABP1 expression was strongly correlated with late tumor stages, arterial involvement, lymph node metastasis, CA 19 – 9 levels and poor overall survival in patients with PDAC [24]. In other cancer types, including gastric, breast and ovarian cancer, increased HABP1 expression has been associated with worse patient outcome [26, 28, 33–38]. These findings suggest that HABP1 could be a promising prognostic marker in patients with PDAC and other cancers.

Regarding therapeutic implications, HABP1 could be a promising therapeutic target for PDAC. Interestingly, high HABP1 expression correlates with better survival in patients with malignant pleural mesothelioma who received neoadjuvant or adjuvant chemotherapy [39]. This suggests that high HABP1 expression could be used as a biomarker to predict the response to chemotherapy. In the present study, however, the association between high HABP1 expression and response to chemotherapy was unclear because of the limited number of patients. Further studies are required to elucidate the relationship between HABP1 expression and chemosensitivity in PDAC.

In conclusion, we demonstrate that HABP1 is overexpressed in PDAC and its expression correlates with prognosis. We also found that HABP1 is involved in proliferation, colony formation, and migration of PDAC cells. These findings suggest that HABP1 may play a role in progression of PDAC.

Abbreviations

CA 19 – 9, carbohydrate antigen 19 – 9; CEMIP, cell migration-inducing and hyaluronan-binding protein; ECM, extracellular matrixes; gC1qR, globular head receptor for complement component 1q; HMW-HA, high-molecular-weight hyaluronan; HPDE, human pancreatic duct epithelial cells; HA, hyaluronan; HABP1, Hyaluronan-Binding Protein 1; HAS, hyaluronan synthases; HYALs, hyaluronidases; IHC, Immunohistochemistry; LMW-HA, low-molecular-weight hyaluronan; ROS, reactive oxygen species; PDAC, pancreatic ductal adenocarcinoma

Declarations

Declarations

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Tables

Due to technical limitations, table 1-2 is only available as a download in the Supplemental Files section.
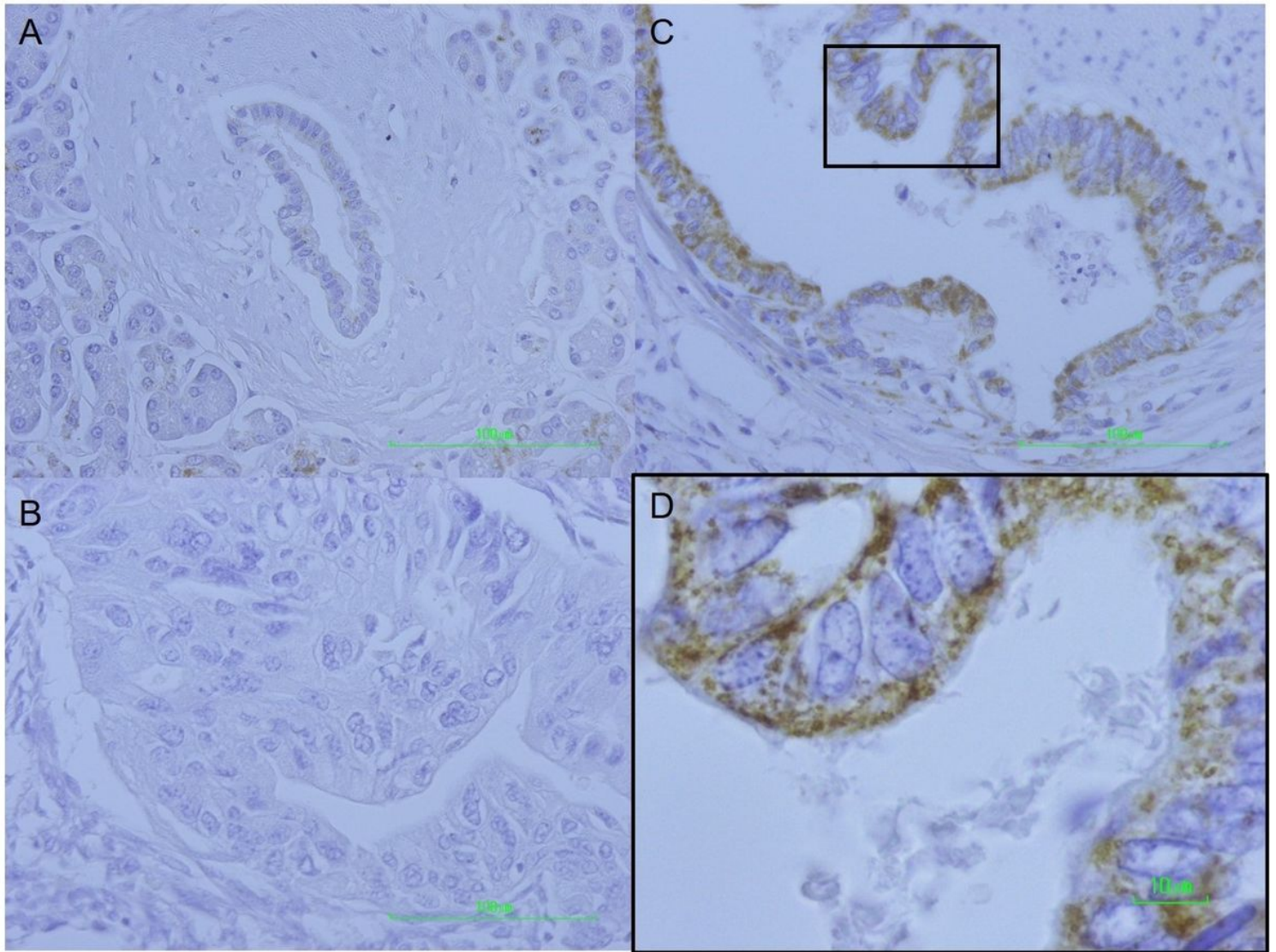
Figures

Figure 1

IHC of PDAC tissue A: a; A normal pancreata including ductal cells (400x, scare bar: 100 μm). b: PDAC tissue of weak HABP1 expression (400x, scare bar: 100 μm). c: PDAC tissue of strong HABP1 expression (400x, scare bar: 100 μm). d: Magnifying image of C (1000x, scare bar: 10 μm). HABP1 expresses in membrane and/or cytoplasm of the tumor cells.
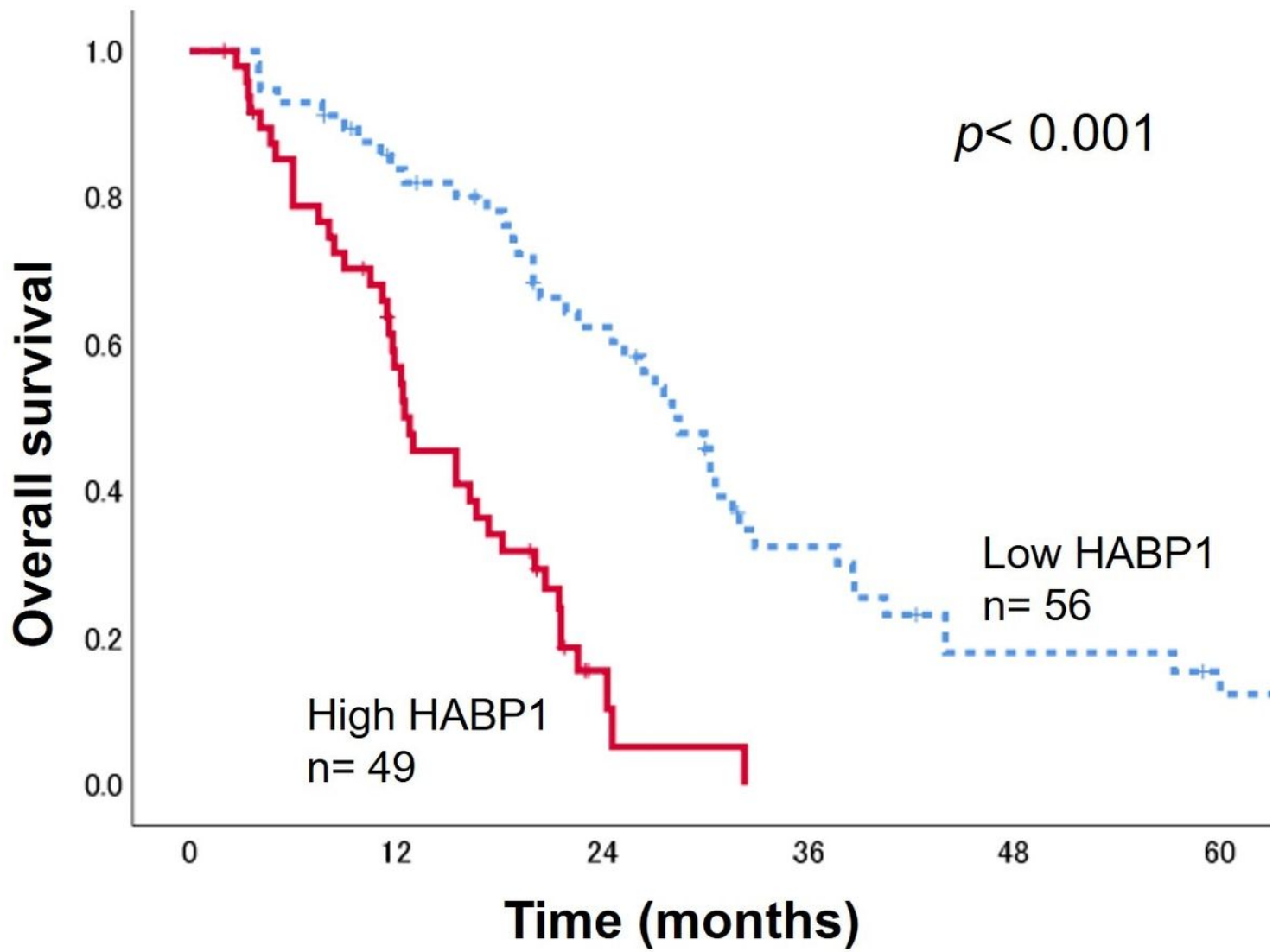
Figure 2

Kaplan-Meier survival curve in patients with PDAC with high HABP1 expression and in those low expression as classified by immunohistochemical staining
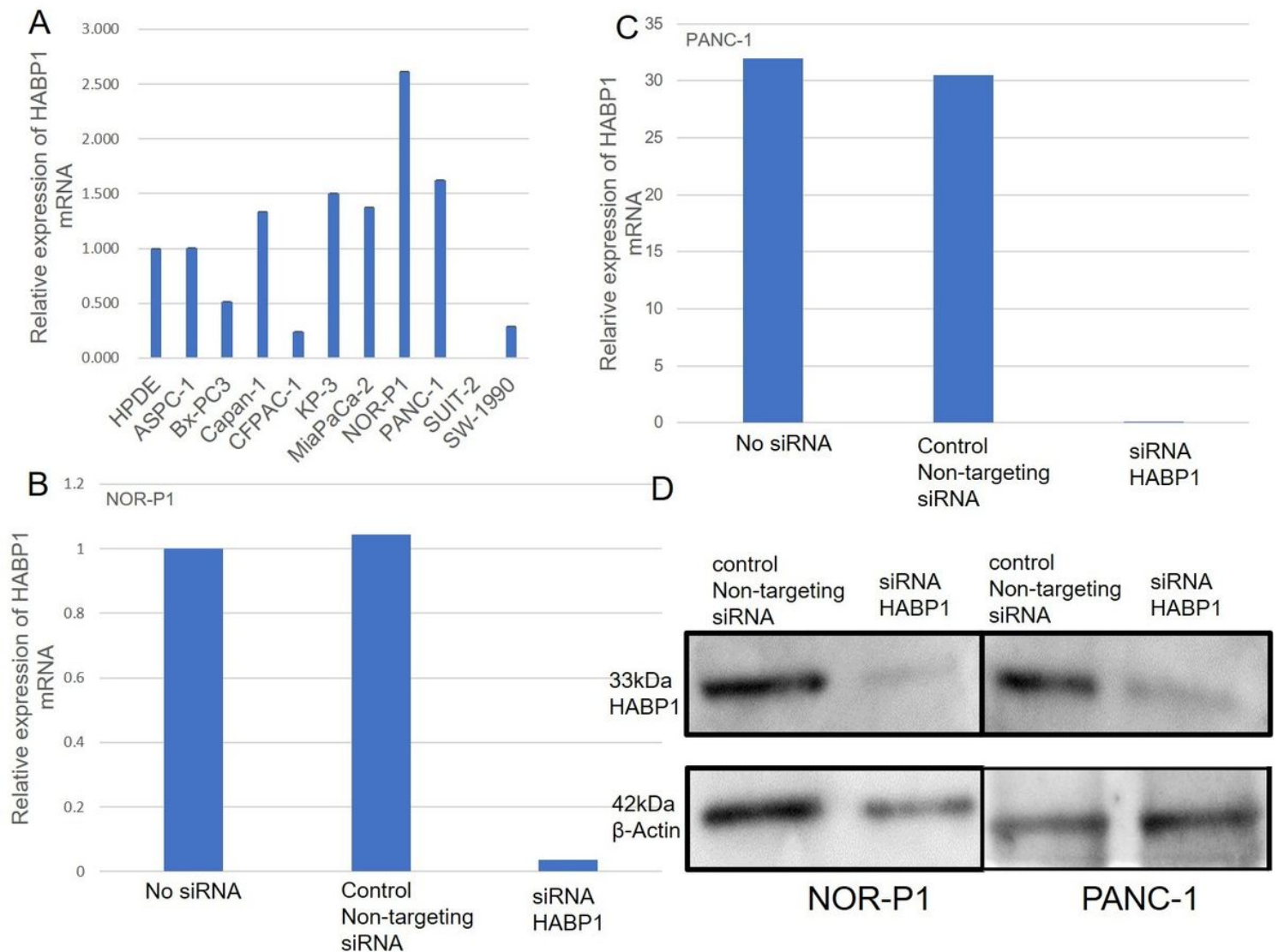
Figure 3

mRNA of HABP1 expression in PDAC cell lines and knockdown of HABP1 used siRNA A: Real-time RT-PCR showing mRNA expression of HABP1 in HPDE and 10 PDAC cell lines. B and C: Real-time RT-PCR showing that transfection with siRNA targeting for HABP1 (siRNA-HABP1) resulted in over 90% knockdown in NOR-P1 and PANC-1. D: Western blot showing that HABP1 protein loss in knockdown group. the samples derive from the same experiment and that blots were processed in parallel.
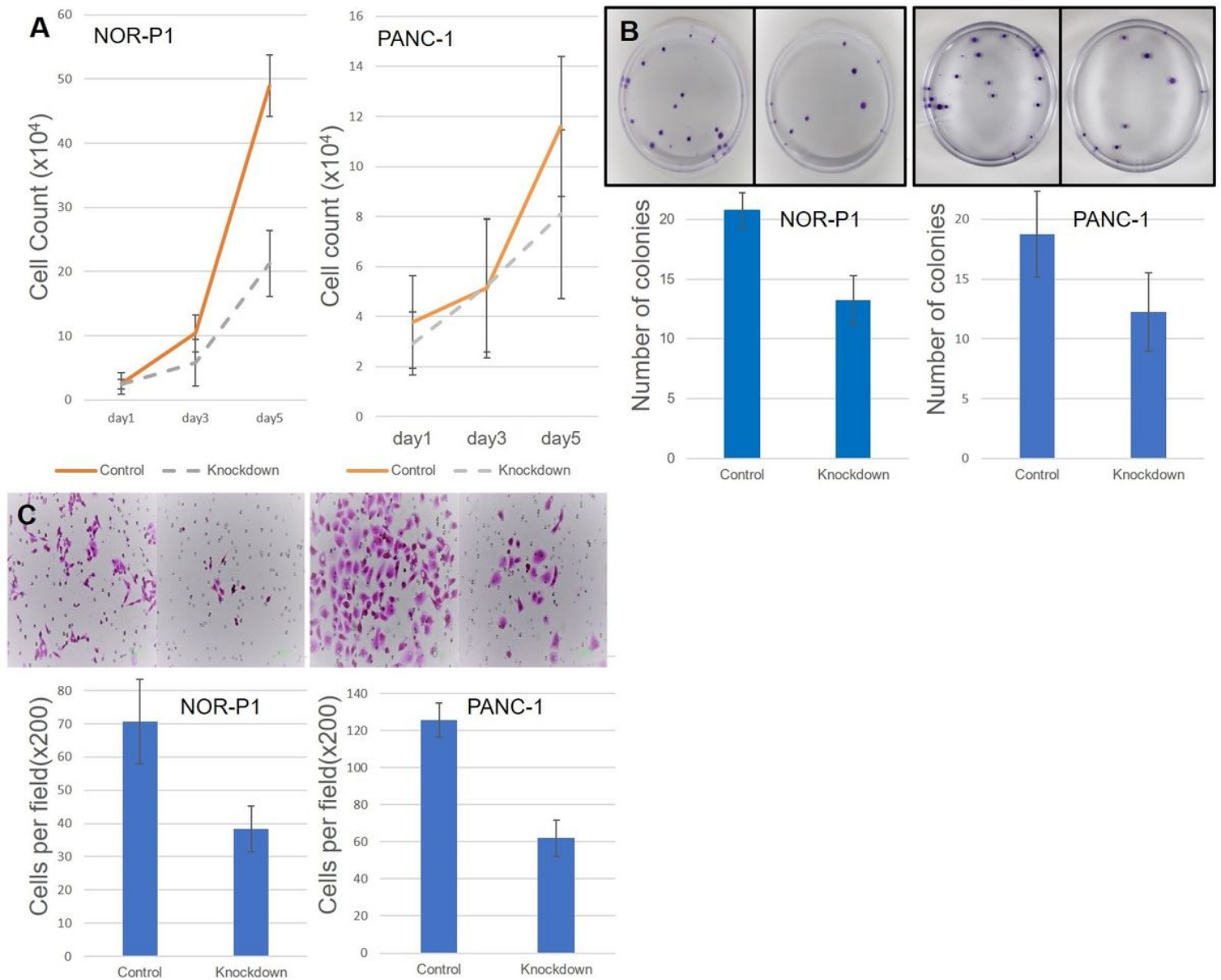
Figure 4

Knockdown of HABP1 suppressing cell malignant behaviors A: At day 5, the cell counting showing knockdown of HABP1 cell numbers are significantly lower than control in NOR-P1 and PANC-1 (n= 3). B: Colony formation assay showing decreased number of colonies in HABP1 suppression group than in control group (n= 3). C: Migration assay showing HABP1 knockdown reduces the number of cell migration (n= 5).

Supplementary Files

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- Table1.xlsx
- Table2.xlsx