Protein kinase, membrane-associated tyrosine/threonine 1 is associated with the progression of colorectal cancer

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Received October 9, 2017; Accepted April 11, 2018

DOI: 10.3892/or.2018.6371

Abstract. The protein kinase, membrane-associated tyrosine/threonine 1 (PKMYT1) is known to inhibit precocious entry into mitosis by phosphorylating CDK1 at Thr14 and Tyr15 residues. However, the functional importance of PKMYT1 in colorectal cancer (CRC) remains unknown. Thus, it is important to elucidate whether PKYMT1 is indispensable in the tumorigenesis of CRC. To investigate the functional importance of PKMYT1 in CRC tumorigenesis, PKMYT1 was knocked down in CRC cell lines such as SW480, SW620, HCT116 and HT29 by siRNA. PKMYT1-depleted CRC cells were analyzed to determine proliferation, migration, invasion and colony forming ability. In addition, 179 patient-derived samples were used to find the correlation of the expression of PKMYT1 with the prognosis of CRC patients. By siRNA-mediated loss of function of PKMYT1, we observed that proliferation, migration, invasion and colony forming ability of CRC cell lines were significantly impaired in the absence of PKMYT1 *in vitro*. Furthermore, by analyzing patient-derived samples, we revealed the association of PKMYT1 with the overall survival rate of CRC patients. These results indicated that PKMYT1 plays an essential oncogenic role in CRC and could serve as a good therapeutic target for the treatment of CRC.

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Key words: PKMYT1, colorectal cancer, prognosis, metastasis

Introduction

Colorectal cancer (CRC) is the third most common type of cancer worldwide, and the fourth leading cause of cancer-related deaths, with an incidence of more than one million new cases per year (1-5). In addition, a significant increase of the incidence rates in CRC in both sexes has been reported, in Korea from 1999 to 2010 (4). The development and progression of CRC occur over several years with distinct molecular and cytological characteristics, eventually leading to a carcinoma with a higher risk of invasion and metastasis (3,6). The prevalent metastasis of CRC is to the lymph node and the liver, which influence approximately half of CRC patients, with a poor survival rate after the diagnosis (7-9). It has been demonstrated that one of the important factors causing progression and poor prognosis of cancer is metastasis (10-12). Though tumor/node/metastasis (TNM) classification and staging systems are widely used to predict prognosis of CRC patients, it remains difficult to precisely predict prognosis (13). Thus, it has been suggested that it is urgently required to identify specific biomarkers to improve the outcomes of patients with CRC (14).

The cell cycle is tightly regulated to prevent premature cell cycle progression, leading to faithful gene replication and segregation (15). Cyclin-dependent kinase 1 also known as CDK1 or cell division cycle 2 (CDC2) is an essential enzyme in cell cycle progression, particularly in the regulation of the G2/M cell cycle transition (16). The activity of CDK1 is regulated by cyclin binding and phosphorylation (17). The phosphorylation of CDK1 at Tyr15 and Thr14 residues leads to the inactivation of CDK1, resulting in a blockade of the transition from G2 to the mitotic phase (17). The protein kinase, membrane-associated tyrosine/threonine 1 (PKMYT1) was originally identified from *Xenopus laevis* as Myt1, a membrane-associated inhibitory kinase that phosphorylates CDK1 efficiently on both threonine-14 and tyrosine-15 (18). Subsequently, it was cloned from human with 46% identification to *Xenopus laevis* PKMYT1 and localization to

the endoplasmic reticulum and Golgi complex (19). Thus, PKMYT1 was demonstrated to inhibit the progression of the cell cycle through the phosphorylation of CDK1. In addition, it was revealed that PKMYT1 was required to induce apoptosis, leading to the suppression of skin cancer development induced by UVA (20). Recently, however, several studies reported that although PKMYT1 is dispensable for the normal cell cycle, it has a rate-limiting function during checkpoint recovery after DNA damage or it is an essential molecule in glioblastoma stem-like cells, thus highlighting a potential role of PKMYT1 as a therapeutic target (21,22). Since the functional importance of PKMYT1 in CRC remains unknown, it is important to comprehend whether PKYMT1 is indispensable in CRC. Thus, using CRC cell lines and patient-derived samples, we addressed these issues and reported that PKMYT1 was essential in the progression of CRC.

Materials and methods

Cell lines. Human CRC cell lines SW480, SW620, HCT116 and HT29 were purchased from the Korean Cell Line Bank (KCLB; Seoul, Korea). Cells were grown in RPMI-1640 medium (Welgene, Geyongsan, Korea) supplemented with 10% fetal bovine serum (FBS; Corning Inc., Corning, NY, USA) and 1% penicillin/streptomycin (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37˚C in a humidified atmosphere containing 5% CO_2.

siRNA and transfection. The siRNAs were obtained from Bioneer Corporation (Daejeon, Korea) (RNAi nos. 1117350, 1117347 and 1117348) and transfected using HiPerFect Transfection reagent (Qiagen, Inc., Valencia, CA, USA) according to the manufacturer's instructions.

RNA extraction and realtime qPCR. RNA was isolated using RiboEx solution (GeneAll, Seoul, Korea), and converted to cDNA using ReverTra Ace® qPCR kit (Toyobo Life Science, Osaka, Japan) according to the manufacturer's instructions. To determine the level of gene expression, RT-qPCR was performed using the qPCR Master Mix kit (Toyobo Life Science). The primer sequences for RT-qPCR are listed in Table I.

Western blot analysis. Cell lysates were harvested using Pro-Prep (Intron Biotechnology Inc., Seongnam, Korea) and centrifuged at 16,000 x g for 10 min at 4˚C. Protein concentration of the supernatant was determined by Bio-Rad Protein assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The protein extract was resolved using 10% polyacrylamide gel and electro-transferred onto a 0.45-μm hybridization nitrocellulose filter (HATF) membrane (Millipore, Billerica, MA, USA) using Trans-Blot Turbo (Bio-Rad Laboratories, Inc.). Membranes were immunoblotted with either mouse anti-PKMYT1 primary antibody (cat. no. NBP2-2275; Novus Biologicals, LLC, Littleton, CO, USA) diluted 1:500 in TBS-T or rabbit polyclonal anti-actin antibody (cat. no. ab179467; Abcam, Cambridge, MA, USA) overnight at 4˚C. The membranes were incubated with either HRP-conjugated anti-rabbit immunoglobulin (cat. no. 7071; Cell Signaling Technology, Inc., Danvers, MA, USA) or HRP-linked anti-mouse immunoglobulin (cat. no. 7076; Cell Signaling Technology, Inc.) for 1 h at room temperature. The protein signal was detected by enhanced chemiluminescence (Thermo Fisher Scientific, Inc., Waltham, MA, USA) using the Amersham Imager 600 (GE Healthcare Life Sciences, Chalfont, UK).

Invasion and migration. Cell migration and invasion were analyzed *in vitro* using the Transwell insert system (Corning Incorporated) without or with coating by 20 μl of Matrigel (BD Biosciences, Franklin Lakes, USA). The culture insert was attached on the bottom of a 24-well plate and 100 μl of serum-free medium containing $1.0x10^5$ cells was seeded into each well. Six-hundred microliters of media containing 10% FBS were added outside the Transwell culture insert. Cells were incubated at 37˚C for 48 h in a humidified atmosphere with 5% CO_2. Transwells were washed twice with phosphate-buffered saline (PBS) and cleaned using cotton swaps. The cells were fixed with 1% formaldehyde for 15 min, washed twice with PBS, stained with 0.1% of crystal violet for 15 min and then observed using an inverted microscope (Leica Microsystems GmbH, Wetzlar, Germany).

Semi-solid agar colony forming assay. Cell culture media containing 20% FBS was mixed with 1% agarose at 1:1 ratio and was solidified after dropping a few drops. After mixing the cell-containing media with agarose to make 0.35% agarose, cells were spread and incubated for 17 days at 37˚C in 5% CO_2 incubator.

Human colorectal carcinoma specimens. A total of 179 colorectal carcinoma tissue specimens were obtained from Soonchunhyang University Cheonan Hospital (Cheonan, Korea), where samples were collected from patients who underwent surgery between March 2002 and May 2006. This study was conducted in accordance with the ethical standard of the Ethics Committee of Soonchunhyang University Cheonan Hospital. These tissues were formalin-fixed and paraffin-embedded (FFPE). Clinicopathological data including age, sex, TNM classification, invasion of blood vessel and lymphatic vessel are listed at Table II. Tumor stage was identified according to the American Joint Committee on Cancer's TNM classification system.

Tissue microarray (TMA) and immunohistochemistry (IHC) assay. Immunohistochemical staining was performed using tissue microarray (TMA) block sections to determine the expression of PKMYT1 in CRC patient samples. Each TMA block contained 60 cores (2 mm of size) from 30 samples. For immunohistochemistry, 4-μm sections were obtained using a microtome, deparaffinized in xylene and rehydrated in 100-70% alcohol series. Antigen retrieval was achieved in citrate buffer (pH 6.0) using a microwave for 15 min. To eliminate endogenous peroxidase activity, the sections were incubated in peroxidase blocking solution (Dako; Agilent Technologies GmbH, Waldbronn, Germany) for 30 min and then washed with PBS containing 0.1% Tween-20 (PBST). The sections were incubated with anti-PKMYT1 antibody (1:10 dilution; cat. no. NBP2-2275; Novus Biologicals, LLC) for 2 h at room temperature, followed by a secondary antibody for 1 h

Table I. Primer sequences.

Name	Sequence	Assay
PKMYT1 primer forward	5'-GGAGAACTGGGTTAAGATGC-3'	RT-PCR
PKMYT1 primer reverse	5'-TCCACTTCCAGAATGGCAGT-3'	RT-PCR
GAPDH primer forward	5'-CTTAGCACCCCTGGCCAAG-3'	RT-PCR
GAPDH primer reverse	5'-GATGTTCTGGAGAGCCCCG-3'	RT-PCR

PKMYT1, protein kinase, membrane-associated tyrosine/threonine 1.

Table II. Clinicopathological features in patients with PKMYT1 expression.

	PKMYT1 expression			
Clinicopathological factors	Low (N=94)	High (N=85)	Total (N=179)	P-value
Age, years				0.256
<60	25 (26.6)	30 (25.3)	55 (30.7)	
≥60	69 (73.4)	55 (64.7)	124 (69.3)	
Sex				0.449
Male	37 (39.4)	39 (45.9)	103 (57.5)	
Female	57 (60.6)	46 (54.1)	76 (42.5)	
Vascular invasion				1.000
No	75 (79.8)	68 (80.0)	143 (79.9)	
Yes	19 (20.2)	17 (20.0)	36 (20.1)	
Lymphatic invasion				0.180
No	64 (68.1)	66 (77.6)	130 (72.6)	
Yes	30 (31.9)	19 (22.4)	49 (27.4)	
Perineural invasion				0.542
No	87 (82.6)	81 (95.3)	168 (93.9)	
Yes	7 (7.4)	4 (4.7)	11 (6.1)	
Stage				0.455
I, II	45 (47.9)	46 (54.1)	91 (50.8)	
III, IV	49 (52.1)	39 (45.9)	88 (49.2)	

PKMYT1, protein kinase, membrane-associated tyrosine/threonine 1.

30 min at room temperature. After being washed with PBST, the sections were incubated with DAB and observed under a light microscope.

IHC data analysis. The PKMYT1-stained tissue cores were examined and a consensus score was determined for each specimen. A positive reaction was scored into 4 grades, according to the intensity of the staining as follows: 0, 1+, 2+ and 3+. The percentages of PKMYT1-positive cells were also scored into 4 categories as follows: 0, 0; 1, 1-30; 2, 31-70; and 3, 71-100%. The final score, calculated as the product of the intensity score multiplied by the percentage score, was classified as follows: 0 for negative; 1-3 for weak; 4-6 for moderate; and 7-9 for strong. Samples with a final score ≤3 were grouped together as PKMYT1-expression negative while those with a score ≥4 were grouped together as PKMYT1-expression positive.

Statistical analysis. Statistical analysis was conducted using SPSS 19.0 (IBM Corp., Inc., Armonk, NY, USA) program. The results of RT-qPCR, migration and invasion were analyzed with Student's t-test. Hazard ratio and 95% confidence interval of clinicopathological data were evaluated using Cox regression models. Kaplan-Meier method was used to analyse disease-free survival rate using the log-rank test. A P-value of <0.05 was considered to indicate statistically significant differences.

Results

siRNA-mediated knockdown of PKMYT1. Since it was revealed that PKYMT1 may be an interesting target in an anticancer therapy (21,22), we tried to determine whether PKMYT1 was functionally important in CRC using siRNA. CRC cell lines including SW480, SW620, HCT116 and HT29

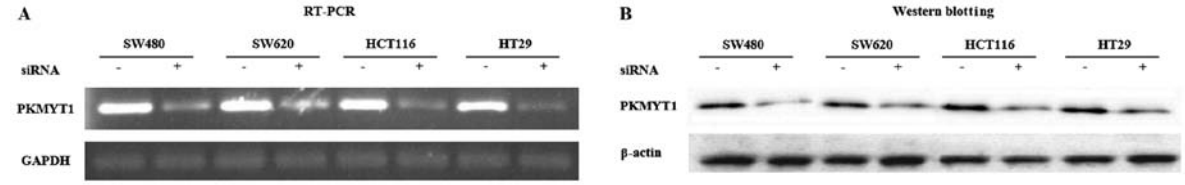

Figure 1. siRNA-mediated PKMYT1 depletion in CRC cell lines. The CRC cell lines (SW480, SW620, HCT116 and HT29) were transfected with either control or PKMYT1 siRNA. The expression of PKMYT1 was determined at the RNA and protein level. (A) RT-PCR was conducted to determine PKMYT1 mRNA expression in cancer cell lines. (B) Immunoblotting with anti-PKMYT1 antibody was performed to analyze PKMYT1 protein level. GAPDH and β-actin were used as the loading control for RT-PCR and immunoblot, respectively. PKMYT1, protein kinase, membrane-associated tyrosine/threonine 1; CRC, colorectal cancer.

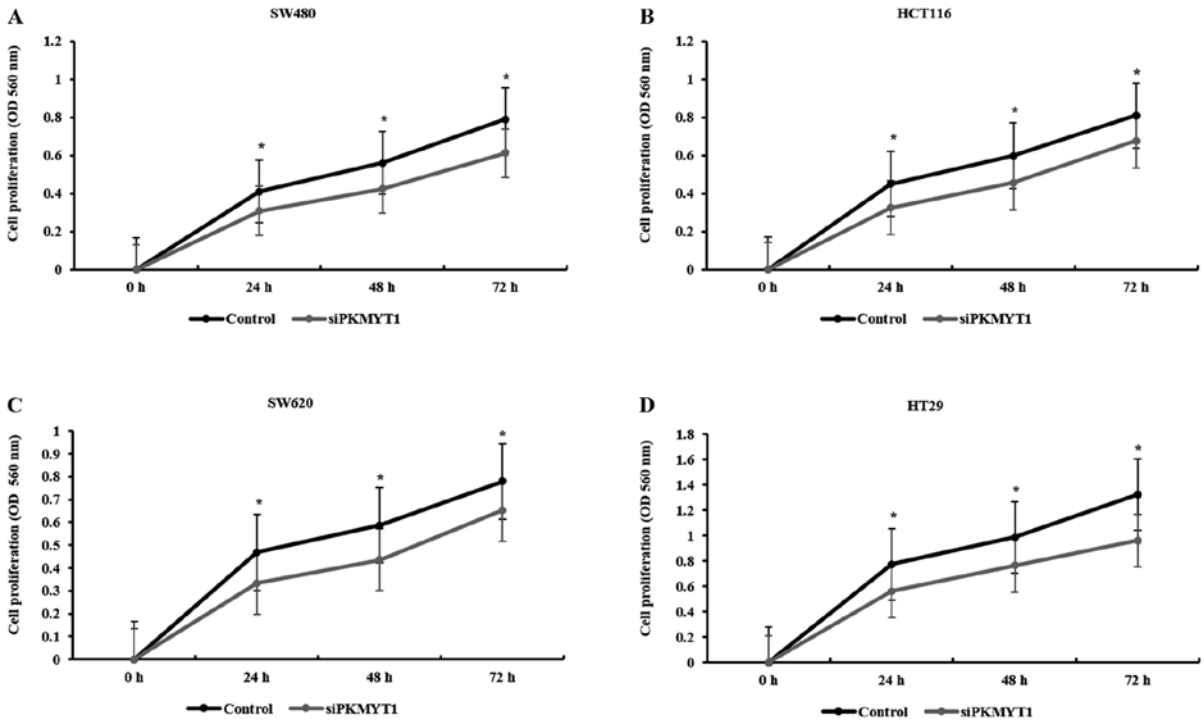

Figure 2. PKMYT1 depletion impairs cell proliferation in CRC cell lines. Control or PKMYT1-knocked down (siPKMYT1) CRC cell lines such as (A) SW480, (B) HCT116, (C) SW620 and (D) HT29 were analyzed every 24 h for 3 days to assess the growth rate. Data shown are from two to three experiments (*P<0.05). PKMYT1, protein kinase, membrane-associated tyrosine/threonine 1. CRC, colorectal cancer.

were transduced with either control or PKMYT1 siRNA. To determine the expression level of *PKMYT1*, RNA was extracted from transduced cells. The expression level of *PKMYT1* was significantly lower in PKMYT1-inhibited cells by siRNA compared to control-transduced cells (Fig. 1A). Since mRNA expression is not always consistent with protein level, we analyzed the level of PKMYT1 protein after siRNA-mediated PKMYT1 knockdown. Immunoblot analysis using cell lysates obtained from siRNA-transduced cells revealed that PKMYT1 siRNA-transduced CRC cell lines expressed much lower level of PKMYT1 protein compared to the control cells (Fig. 1B). From these data, we confirmed that the expression of PKMYT1 was significantly inhibited by siRNA-mediated depletion at both the RNA and protein level.

The proliferation and colony-forming ability of CRC cell lines are defective by the knockdown of PKMYT1. We tested whether PKMYT1 was implicated in the growth of CRC cells using MTT assay after PKMYT1 knockdown. The proliferation rate of control or PKMYT1 siRNA-transfected SW480, HCT116, SW620 and HT29 cell lines was analyzed every 24 h for 3 days. The proliferation rate of PKMYT1-depleted CRC cells was significantly decreased starting from 24 h up to 72 h in the tested CRC cell lines (Fig. 2). To check the anchorage-independent growth of CRC cell lines after knocking down PKMYT1, semi-solid soft-agar colony forming ability was tested. Control or PKMYT1 siRNA-transfected CRC cell lines were seeded on soft-agar, and colony number and size were determined. The colony size in PKMYT1-knockdown cells was much smaller than that of control cells (Fig. 3). Notably, as displayed in Fig. 3, the number of colonies was significantly decreased in PKMYT1-siRNA transfected CRC cell lines compared to the control (SW480, 161 vs. 106; SW620, 211 vs. 87; HCT116, 175 vs. 83; HT29, 190 vs. 101).

PKMYT1 is required for the mobility of CRC cell lines. We examined the mobility change after knocking down PKMYT1 in CRC cells to determine whether PKMYT1 was essential

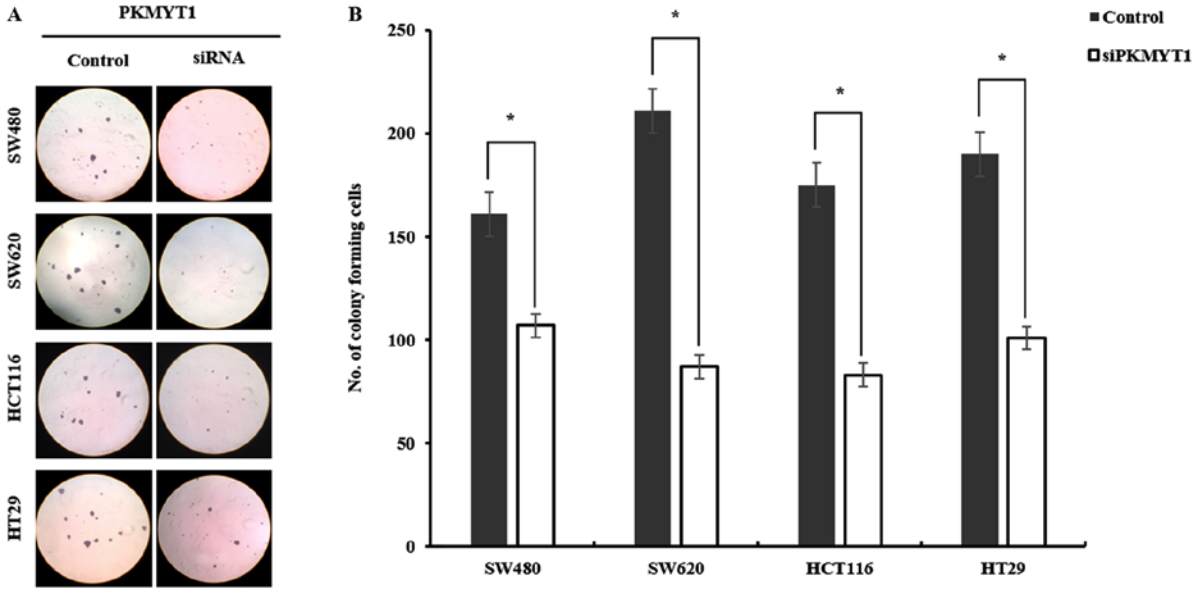

Figure 3. PKMYT1 depletion impairs anchorage-independent colony forming ability of CRC cell lines *in vitro*. Control or PKMYT1-depleted (siPKMYT1) CRC cell lines (SW480, HCT116, SW620 and HT29) were seeded semi-solid agarose, followed by incubation for 21 days to assess colony forming ability. (A) Representative images of colonies are shown. (B) Number of colonies from each cell line were quantified. Data shown are from three experiments (*P<0.05). PKMYT1, protein kinase, membrane-associated tyrosine/threonine 1; CRC, colorectal cancer.

Table III. Univariate and multivariate Cox regression analysis of the relative risk of death according to the expression of PKMYT1.

	Univariate analysis		Multivariate analysis	
Clinicopathological factors	Hazard ratio (95% Cl)	P-value	Hazard ratio (95% Cl)	P-value
Age, years (<60, ≥60)	1.158 (0.721-1.862)	0.544	1.261 (0.768-2.070)	0.359
Sex	1.154 (0.749-1.777)	0.516	1.095 (0.702-1.708)	0.688
Vascular invasion	1.503 (0.908-2.488)	0.113	0.817 (0.396-1.686)	0.584
Lymphatic invasion	2.055 (1.311-3.221)	**0.002**	1.821 (0.923-3.590)	0.084
Perineural invasion	4.219 (2.158-8.245)	**0.000**	3.496 (1.701-7.186)	**0.001**
Clinical stage (I, II, III, IV)	1.817 (1.172-2.817)	**0.008**	1.720 (1.084-2.727)	**0.021**
PKMYT1	1.657 (1.064-2.579)	**0.025**	1.594 (1.020-2.492)	**0.041**

P-values in bold indicate a statistically significant difference. PKMYT1, protein kinase, membrane-associated tyrosine/threonine 1.

in the progression of CRC. CRC cell lines SW480, SW620, HCT116 and HT29 were transfected with either control or PKMYT1 siRNA. Transduced cells were seeded on the Transwell system and incubated for 48 h to assess the migratory ability. The migration of cancer cells was evaluated by counting the cells on the inserts of the Transwell system after staining. PKMYT1 siRNA-transfected cells migrated ~14-38% less than control-transduced cells (Fig. 4A and B). To examine whether PKMYT1 was essential for the invasion of cancer cells *in vitro*, control or PKMYT1-knockdown cells were plated on a Matrigel-coated insert of a Transwell system and incubated for 48 h. PKMYT1 siRNA-transfected cells exhibited an invasion ability ~25-40% less than the control-transduced cells (Fig. 4C and D). These results indicated that PKMYT1 is indispensable for the mobility of CRC cells, thus possibly contributing to the progression of CRC.

The association of PKMYT1 with a poor prognosis of CRC patients. Following the characterization of the pivotal function of PKMYT1 in CRC cell lines *in vitro*, we determined the biological relevance of PKMYT1 to a patient prognosis model. The demographic data of the patients analyzed in the present study are provided in Table II. When the correlation of PKMYT1 expression to overall survival (OS) rate was analyzed, we observed that PKMYT1 was closely associated with the OS rate of CRC patients (Fig. 5). Specifically, patients with a high level of PKMYT1 expression had significantly lower overall survival (OS) rate than patients with low expression of PKMYT1 (45.7 vs. 62.4%, P=0.023; Fig. 5C). In addition, PKMYT1 positivity was correlated with other clinicopathological variables by Cox regression analysis including lymphatic invasion, perineural invasion and clinical stage (Table III). These results indicated the

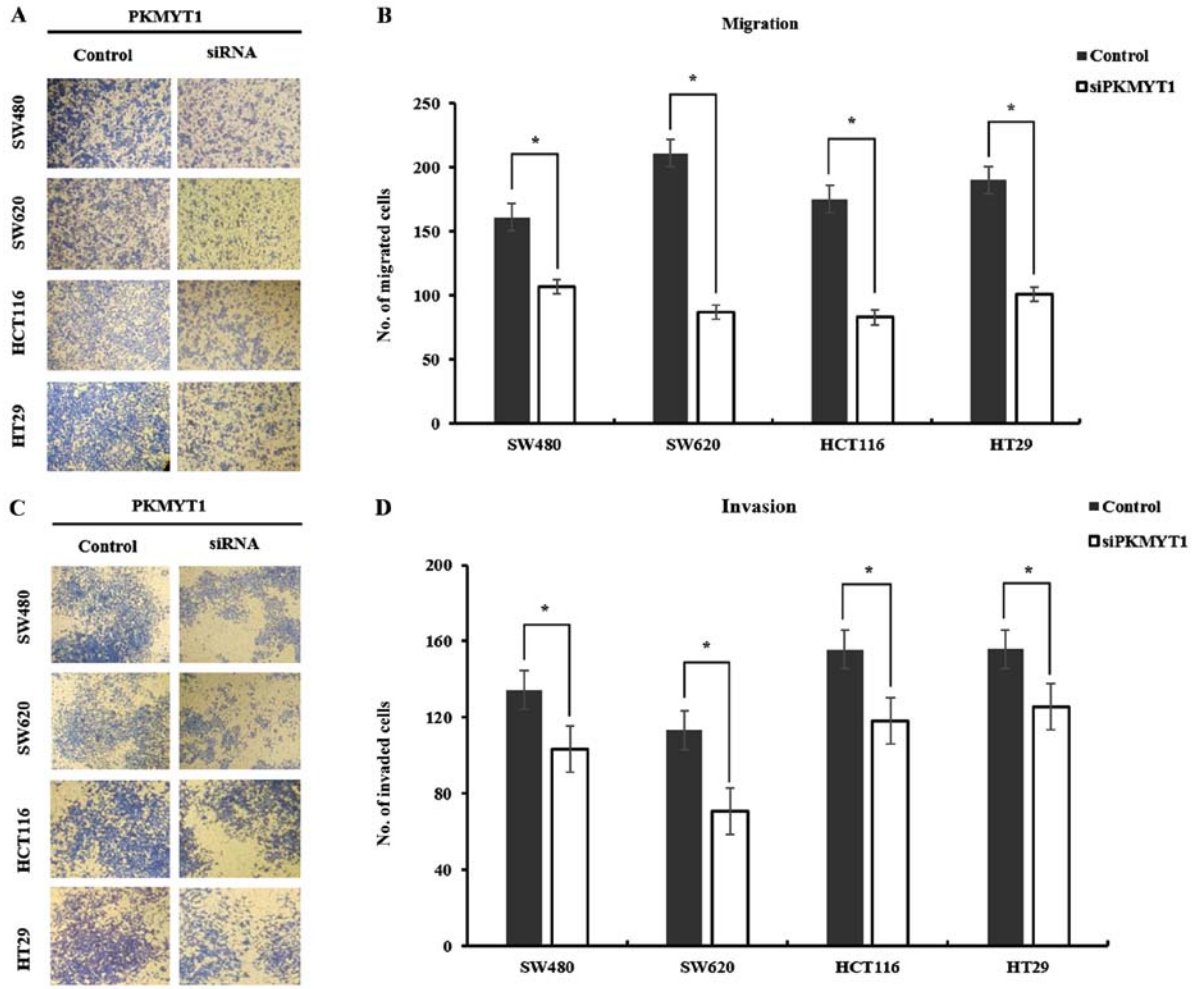

Figure 4. PKMYT1-knockdown CRC cells display impaired mobility *in vitro*. The migration and invasion of control (shLacZ) or PKMYT1-depleted (siPKMYT1) CRC cell lines were determined on a Matrigel-uncoated and coated Transwell, respectively. After incubation for 48 h on an insert of Transwell, cells that had migrated to the lower surface of the Transwell chamber were stained and quantified. The data of migration (A and B) and invasion (C and D) are from three experiments and representative images are displayed (*P<0.05). PKMYT1, protein kinase, membrane-associated tyrosine/threonine 1; CRC, colorectal cancer.

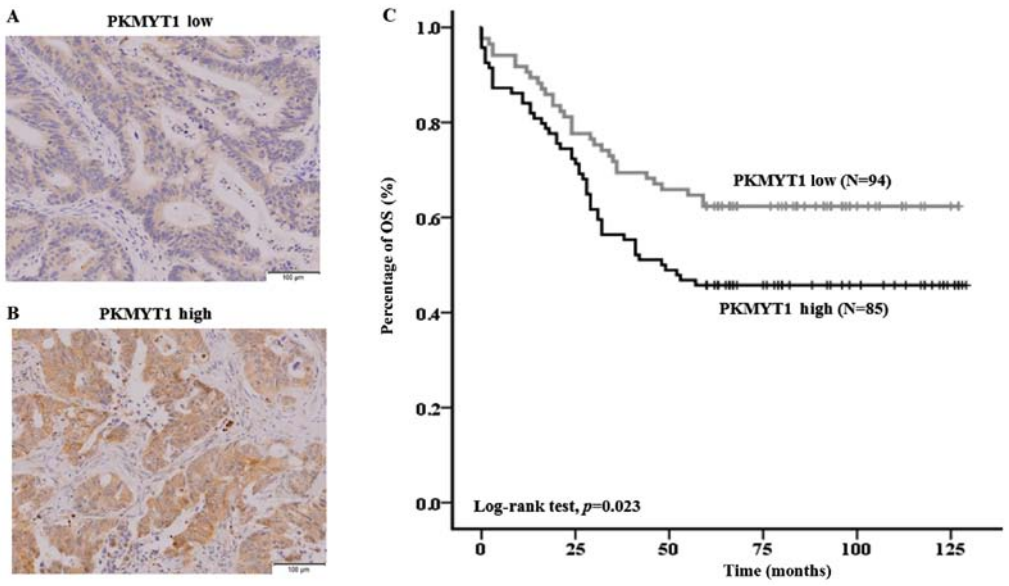

Figure 5. The association of PKMYT1 expression with poor prognosis of CRC patients. To determine the association of PKMYT1 expression with prognosis of CRC patients, 179 CRC samples stained with PKMYT1 antibody were graded based on both staining intensity and frequency. (A and B) Representative images are shown. Strong expression of PKMYT1 protein in a CRC tissue appeared as brown particles which were localized within the nucleus and cytoplasm of epithelial cells. Original magnification, x200. Scale bar, 100 μm. (C) Patients were divided into two groups based on the expression of PKMYT1 and survival rate was determined using Kaplan-Meier analysis (P<0.05). PKMYT1, protein kinase, membrane-associated tyrosine/threonine 1; CRC, colorectal cancer.

close association of PKMYT1 with poor prognosis of CRC patients.

Discussion

In the present study, we found that PKMYT1 was essential for the proliferation and mobility of CRC cells *in vitro*. Depletion of the expression of PKMYT1 by siRNA resulted in impaired proliferation, anchorage-independent colony forming ability and mobility of CRC cells. In addition, using patient-derived samples, we demonstrated that patients expressing a high level of PKMYT1 displayed a worse overall survival rate than those with a low level of PKMYT1. Thus, these results indicated that PKMYT1 was a biomarker for the prediction of the prognosis of the disease. In agreement with our findings, several other studies have also recently reported that PKMYT1 was essential in several cancers such as glioblastoma, however not in normal cells and a promising target in developing chemotherapeutics (21,22).

It is well established that CDK1 is a key molecule in the progression of mitosis and becomes inactivated when phosphorylated by WEE1 (23,24). Although CDK1 is also phosphorylated by PKMYT1, the contribution of PKMYT1 in normal or cancer cells without DNA damage appears relatively minor. Chow *et al* (21) revealed that the cell cycle or mitosis timing in HeLa cells was not affected by the depletion of PKMYT1 without DNA damage. However, PKMYT1 knockdown with DNA damaging agents such as adriamycin or irradiation was able to significantly inhibit cell survival (21). In human neural stem cells, it was demonstrated that PKMYT1 and WEE1 are redundant. However, in glioblastoma cells, depletion of PKMYT1 led to a dramatic increase in cell death and cytokinesis failure (22). Previously, only WEE1 had been studied as a potential therapeutic target in chemotherapy since PKMYT was considered not essential or redundant. However, our data along with other studies indicated that PKMYT1 was essentially required in several cancers including CRC. Furthermore, it is the first study, to the best of our knowledge, to demonstrate that PKMYT1 was implicated in the progression of CRC.

It has been suggested that metastatic spread of CRC is one of the pivotal factors leading to a high level of mortality in CRC patients. Thus, to improve the survival rates of CRC patients, it is required to identify essential signaling pathways implicated in metastasis (25,26). Epithelial-mesenchymal transition (EMT), an important process in tumor progression, is involved in several aspects including invasion, metastasis and drug resistance (27-34). In hepatocellular carcinoma cells, PKMYT1 has been shown to regulate EMT by activating beta-catenin/TCF signaling (35). In the present study, we also found that depletion of PKMYT1 impaired the mobility of CRC cells, a function that is related to EMT, proposing that PKMYT1 was an essential molecule in the progression of CRC. Thus, developing small molecules that block the function of PKMYT1 could be an effective way to inhibit the progression of the disease.

Acknowledgements

Not applicable.

Funding

The present study was supported by the Soonchunhyang University Research Fund and the grant of the Korea Health Technology R&D Project through the Korea Health Industry Development Institute (KHID), funded by the Ministry of Health & Welfare, Republic of Korea (grant no. HI15C1647).

Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

DJ, HYK and MJB conceived and designed the study. DJ, HK, DK, SB, SO, HL, DK, TSA, SBB, MSL and SJ performed the experiments. HYK and MJB wrote the paper. HJK and CJK reviewed and edited the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

All experimental protocols were approved by the Ethics Committee of Soonchunhyang University Cheonan Hospital and Soonchunhyang University Institutional Animal Care and Use Committee.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no conflict of interest.

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