学 位 論 文

学位論文名

Clinical significance of serum *Wisteria floribunda* agglutinin-positive Mac-2 binding protein in pancreatic ductal adenocarcinoma

$\Big($膵管癌における血清 Wisteria floribunda agglutinin-positive Mac-2 binding protein

の臨床的意義の検討$\Big)$

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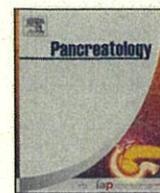消化器内科学講座

藁谷　雄一

論　文　内　容　要　旨（和文）

学位論文題名	Clinical significance of serum *Wisteria floribunda* agglutinin-positive Mac-2 binding protein in pancreatic ductal adenocarcinoma （膵管癌における血清 Wisteria floribunda agglutinin-positive Mac-2 binding protein の臨床的意義の検討）

【目的】Wisteria floribunda agglutinin-positive mac2 binding protein（WFA⁺-M2BP）は M2BP の糖鎖修飾異性体であり，肝線維化の程度に相関する有用なマーカーとして使用されている．本検討では，線維化の豊富な慢性膵炎及び膵管癌における血清中 WFA⁺-M2BP の臨床的意義について検証した．

【方法】慢性膵炎患者群 16 名（CP 群），膵管癌患者群 24 名（PDAC 群）を対象とし，2 群間で既存の腫瘍マーカー，血清 M2BP，血清 WFA⁺-M2BP を比較した．次に WFA⁺-M2BP 陽性および陰性の膵管癌患者の患者背景（腫瘍径，臨床病期，腫瘍マーカー値，予後など）を比較した．さらに，WFA⁺-M2BP の分泌源を検証すべく，陰性コントロール HEK293，既存のヒト膵管癌細胞株（Panc-1，MiaPaCa-2，BxPC-3），膵管癌肝転移巣直接移植マウスモデルより作成された細胞株（MDA-PATC53），不死化ヒト膵星細胞（hPSC21-S/T）を用い，細胞及び培養上清中の WFA⁺-M2BP を測定した．WFA⁺-M2BP の腫瘍細胞に対する生理作用について，培養上清を用いた細胞遊走能，細胞浸潤能を行った．また，WFA⁺-M2BP 陽性細胞と陰性細胞における糖転移酵素群の遺伝子発現の差異を，cDNA マイクロアレイを用いて検証した．なお，WFA⁺-M2BP は cut-off-index（COI）で表記し，肝線維化に準じて 1.0 を cut-off 値とし，1.0 COI 以上を WFA⁺-M2BP 陽性膵癌と定義した．全ての結果は中央値で記載した．

【結果】CP 群と PDAC 群の比較では M2BP（3732 vs. 4072 ng/ml，$P = 0.35$）に差は見られなかったが，WFA⁺-M2BP（0.51 vs. 0.98 COI，$P = 0.001$）では PDAC 群で有意に高値であった．また，M2BP と WFA⁺-M2BP の数値に相関はなく（$r = 0.11$，$P = 0.58$），WFA⁺-M2BP は独立した膵管癌の診断マーカーである事が示唆された．WFA⁺-M2BP 陽性例は転移症例が多く（91.6% vs. 41.7%，$P = 0.009$），化学療法を施行された症例において生存期間中央値が有意に短期間であった（371.5 vs. 250.0 日間，$P = 0.035$）．細胞実験では MDA-PATC53 のみが WFA⁺-M2BP 陽性であった．しかし，1.0 COI の WFA⁺-M2BP を含む MDA-PATC53 上清は，ヒト膵管癌細胞株の増殖能，遊走能，浸潤能に影響を与えず，WFA⁺-M2BP 自体の生理作用はないと推測された．Panc-1（WFA⁺-M2BP 陰性）と MDA-PATC53（WFA⁺-M2BP 陽性）との間の糖転移酵素の遺伝子発現解析では，Panc-1 に比し MDA-PATC53 でがん増殖促進に関与する GALNT-3 と GALNT-14 が増加し，癌抑制に関連する Neu-1 が減少していた．

【考察】膵管癌患者血清中 WFA⁺-M2BP は良悪性診断及び遠隔転移の予測マーカーとなる可能性が示唆された．癌細胞に対する WFA⁺-M2BP の生理作用は認められず，がんの悪性度に関連する糖転移酵素の発現の変化を間接的に反映したものと推測された．

ELSEVIER

Clinical significance of serum *Wisteria floribunda* agglutinin-positive Mac-2 binding protein in pancreatic ductal adenocarcinoma

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ABSTRACT

Background: Wisteria floribunda agglutinin-positive mac-2 binding protein (WFA$^+$-M2BP) is an excellent biomarker for predicting hepatic fibrosis. We hypothesized WFA$^+$-M2BP might be a serum biomarker for the diagnosis of chronic pancreatitis (CP) and pancreatic ductal adenocarcinoma (PDAC) with dense fibrosis.
Methods: In this study, we included 16 CP and 24 PDAC patients. Serum levels of WFA$^+$-M2BP (cut-off index [COI]) were compared between the 2 groups. To confirm the cellular production of WFA$^+$-M2BP, we investigated the presence of WFA$^+$-M2BP in HEK293 cells, 3 established human PDAC cell lines and a recently generated human PDAC cell line derived from a liver metastasis (MDA-PATC53). The bio-physiological effects of MDA-PATC53 supernatant were evaluated. Finally, the difference in the expression of glycosylation enzymes between MDA-PATC53 and Panc-1 were analyzed by cDNA microarray.
Results: We found that the serum WFA$^+$-M2BP level could distinguish the 2 groups. The median serum COI of WFA$^+$-M2BP was 0.98 and 0.51 in PDAC and CP, respectively. Additionally, WFA$^+$-M2BP positive PDACs were more frequently associated with metastatic lesions than the WFA$^+$-M2BP negative PDACs (91.6% vs. 41.7%, $P = 0.009$). The MDA-PATC53 cells alone produced WFA$^+$-M2BP. However, we found that MDA-PATC53 supernatant containing WFA$^+$-M2BP (1.0 COI) did not alter the biological behavior of cancer cell lines. The results of cDNA microarray revealed that several glycosylation enzymes with pro-oncologic function were highly expressed in MDA-PATC53 compared to Panc-1.
Conclusions: Serum WFA$^+$-M2BP can be a useful biomarker for the diagnosis of PDAC and the prediction of disease progression since it potentially reflects altered pro-oncologic glycosylation enzymes.
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1. Introduction

Pancreatic ductal adenocarcinoma (PDAC) has one of the highest case fatality rates of cancer in the world [1]. PDAC is often associated with metastatic lesions in other organs, even when it is located within the pancreatic parenchyma [2]. To confront this challenging issue, researchers have attempted to identify biomarkers for the detection of PDACs, and some of these markers (e.g., serum tumor markers [3–6], micro RNA [7–9], cell free DNA [10], etc.) are thought to be clinically useful in specific situations.

Recently, an assay that detects *Wisteria floribunda* agglutinin-positive Mac-2 binding protein (WFA$^+$-M2BP) was developed as a novel and less-invasive method for assessing liver fibrosis [11]. Moreover, the presence of WFA$^+$-M2BP can predict the existence of hepatocellular carcinomas in cirrhotic patients [12,13]. Because chronic pancreatitis (CP), which is one of the significant risk factors for the development of PDAC, and PDAC itself are associated with dense fibrosis, we hypothesized that the serum levels of WFA$^+$-M2BP associated with these conditions might be altered in a manner similar to that observed in liver cirrhosis and concomitant

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carcinoma.

In this report, we first compared the serum levels of normal M2BP and WFA⁺-M2BP in volunteers who were controls, and patients with CP or PDAC to clarify the clinical significance of these markers in terms of the differentiations of PDAC and other conditions. In a second step, we performed an in vitro study to confirm the origin of WFA⁺-M2BP in several cell lines, including human pancreatic cancer cell lines derived from primary and metastatic sites.

2. Methods

2.1. Patients and sample collection

Between April and December 2015, we prospectively enrolled CP and PDAC patients. CP was diagnosed on the basis of imaging studies including computed tomography or ultrasounds with typical findings (e.g., calcification and pancreatic duct dilatation). All PDAC patients underwent endoscopic ultrasound-guided fine-needle aspiration biopsy (EUS-FNA) and the pathology confirmed. Patients with histories of chronic liver disease were excluded because such diseases can affect the serum level of WFA⁺-M2BP as previously described [12,13]. Informed consent was obtained from all patients. The study protocol was approved by the Institutional Review Board of Fukushima Medical University.

The patients' clinical data, including age, sex, serum carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9 (CA 19-9) levels were obtained from electronic medical records. Biological data regarding the tumors (e.g., tumor size and the location of the tumor) were also obtained. Blood samples were collected after obtaining informed consent and were immediately processed to separate the serum and stored at −20 °C.

Among patients who underwent gemcitabine-based chemotherapy, overall survival was calculated from the day of diagnosis by EUS-FNA to death or the last follow-up examination.

2.2. Cell culture

Three commercial PDAC cell lines (Panc-1, MiaPaCa-2, and BxPC-3) and immortalized human embryonic kidney 293 cells (HEK293T) were obtained from ATCC. The MDA-PATC53 cell line, which was recently generated from a direct xenograft PDAC, was kindly provided by Dr. Jason B. Fleming [14].

The immortalized human pancreatic stellate cell line hPSC21-S/T was established by the introduction of simian virus 40 T antigen and human telomerase reverse transcriptase into the human primary PSCs [15]. The hPSC21-S/T cells expressed typical stellate cell markers including α-smooth muscle actin, vimentin, type I collagen, and glial fibrillary acidic protein [16]. The hPSC21-S/T cells were grown in DMEM supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 4.5 g/L glucose, 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. Other cell lines were grown in RPMI-1640 with the same supplements. All cell lines were cultured in a humidified atmosphere containing 5% CO_2 at 37 °C and were grown to 70%–80% confluence in 10 cm culture dishes in medium before further processes.

2.3. Preparation of samples from cell culture

To obtain cell lysates, the cells were prepared by rinsing twice with PBS and scraping with RIPA buffer (50 mM Tris HCl, pH 8, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS)

supplemented with a protease inhibitor cocktail (Thermo Fisher Scientific). Total RNA from cells was purified using the RNeasy mini kit (Qiagen) according to the manufacturer's protocol. The cDNA was prepared from 1 μg total RNA by using a RT2 PCR array first strand kit (Qiagen). To obtain supernatant, the medium was changed to serum-free RPMI and DMEM medium, and the cells were cultured for 24 h. The supernatant was collected, filtered through a 0.45-μm filter and concentrated with an Amicon Ultra-15 centrifugal filter unit with an ultracel-30 membrane (Millipore). For further experiments, supernatant from MDA-PATC53 cell lines was diluted with phosphate saline buffer to have a cut-off index [COI] of 1.0 WFA⁺-M2BP.

2.4. Measurement of the M2BP and WFA⁺-M2BP levels

The serum level of M2BP was measured with an ELISA kit (Abcam, Cambridge, MA) according to the manufacturer's protocol. The levels of WFA⁺-M2BP in serum, cell supernatant and cell lysate were measured with a sandwich immunoassay as previously described. Briefly, glycosylated M2BP was captured by WFA that was immobilized on magnetic beads. The bound product was assayed with an anti-human M2BP monoclonal antibody linked to alkaline phosphatase. The assay manipulation was fully automated using a chemiluminescence enzyme immunoassay machine (HISCL-2000i; Sysmex, Kobe, Japan). All counts were standardized and converted to a COI designated as WFA⁺-M2BP [11,13]. When performing in vitro experiments, WFA⁺-M2BP levels greater than 1.0 COI were considered to be positive.

2.5. MTT cell proliferation assay

Panc-1, MiaPaca-2 and BxPC-3 cells were seeded at a density of 3000 cells in 200 μl media with 10% FBS in a 96-well plate. After 24 h of incubation, the media was exchanged to RPMI-1640 media without any FBS [FBS (-)], RPMI-1640 media with 10% FBS [FBS (+)], and MDA-PATC53 conditioned supernatant [FBS (-) PATC53] and incubated for 48 h. MTT (5 mg/ml) was added to each well, and the optical density of each well was measured at 570 nm by using a Benchmark Plus microplate spectrophotometer (Bio-Rad Laboratories, Hercules, CA).

2.6. Cell migration and invasion assay

The cell migration assay was evaluated using 8.0 μm pore size Millicell Hanging Cell Culture Inserts (Millipore). After pre-incubation with RPMI-1640 medium for 30 min at 37 °C, the lower chambers were filled with 650 μl of RPMI-1640 medium containing 10% FBS, medium without FBS and MDA-PATC53 conditioned supernatant. Subsequently, PDAC cells (Panc-1, MiaPaca-2, BxPC-3) with a density of 1×10^5 cells per well in 500 μl RPMI-1640 medium without FBS were seeded into the inserts, and allowed to trans-migrate for 24 h at 37 °C in 5% CO^2 atmosphere. Invasion assays were conducted in 24-well cell culture dishes using specialized inserts containing an 8 μm pore size polycarbonate membrane (CORNING). Briefly, PDAC cells (2×10^4 per well) in 500 μl of serum free RPMI-1640 medium were seeded in the upper chambers. The lower chambers were filled with 650 μl RPMI-1640 medium containing 10% FBS, medium without FBS and MDA-PATC53 conditioned supernatant. The cells were allowed to invade the Matrigel for 48 h. After incubation, non-migrated and invaded cells on the upper side of the membrane were scraped off with cotton swabs. Subsequently, migrated and invaded cells were

fixed in menthol, stained with Crystal violet, and counted in 3 adjacent microscopic fields for each membrane at magnification (x 200).

2.7. cDNA microarray

Differences in transcript levels of glycosylation enzymes between Panc-1 and MDA-PATC53 were analyzed using an RT2 Profiler PCR Array (SABiosciences). Quantitative PCR was performed with StepOne Plus (Applied Biosystems) using RT2 Real-Time SYBR Green PCR Master Mix according to the manufacturer's protocol. We chose glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control and Panc-1 as a control group.

2.8. Statistical analyses

Continuous variables (age, tumor size, and serum CEA, CA 19-9, M2BP and WFA$^+$-M2BP levels) are described as the median (range) values and were compared with the Mann-Whitney U test. Sex, locality of disease, and the Union for International Cancer Control (UICC) cancer stage were compared with Fisher's exact probability tests. The diagnostic performances of WFA$^+$-M2BP and CA 19-9 in the differentiation of PDAC and CP and in detection of metastasis was assessed using receiver operating characteristic (ROC) curves and examining the areas under the ROC curves (AUROCs). The correlations of the serum levels of the tumor markers with WFA$^+$-M2BP were analyzed with Pearson's correlation analyses. Survival analysis was performed using the Kaplan-Meier method with log-rank test. All statistics were performed with GraphPad Prism 6.0 (GraphPad Software, San Diego, CA). Differences were considered statistically significant at $P < 0.05$.

3. Results

Serum WFA$^+$-M2BP was a useful biomarker in the differentiation of PDAC and CP.

During the study period, we included 16 patients with CP and 24 patients with PDAC. The clinical characteristics and morphologic information are summarized in Table 1. CP was frequently observed in male patients ($P = 0.02$). Pancreatic stone and calcification was often observed in CP compared to PDAC ($P = 0.0001$).

The serum M2BP level was not significantly different between the CP and PDAC groups (3732 ng/ml vs. 4072 ng/ml; $P = 0.35$). In contrast, we found that serum WFA$^+$-M2BP levels could be used to distinguish them. The serum level of WFA$^+$-M2BP was 0.98 COI (0.28–2.8) in PDAC and 0.51 COI (0.36–0.93) in CP (Fig. 1A and B). The serum level of WFA$^+$-M2BP was independent of M2BP since correlation analysis did not find a significant difference (Fig. 1C).

Comparative study of the serum biomarkers in the CP and PDAC patients revealed that the serum CA 19-9 and WFA$^+$-M2BP levels were feasible biomarkers that might aid the differentiation of PDAC

and CP, whereas the serum CEA and M2BP levels were not significantly different between these groups (Table 2). ROC curve analyses revealed good diagnostic accuracies of CA 19-9 (AUC = 0.85) and WFA$^+$-M2BP (AUC = 0.76; Table 3).

Serum WFA$^+$-M2BP could be a marker to predict the existence of metastasis and the prognosis in pancreatic cancer patients.

Next, we analyzed the clinical characteristics of WFA$^+$-M2BP-positive and -negative PDACs (Table 4). Among several clinical and biological parameters, we more frequently found WFA$^+$-M2BP positivity among the patients with metastatic lesions (UICC Stage IV) than in the patients with localized lesions (UICC Stage I — III). In ROC curve analysis, we found that WFA$^+$-M2BP could be a potential surrogate marker to predict the existence of metastasis with good specificity and positive predictive value (PPV) (87.5% and 90.9%, respectively, Table 5). Moreover, survival analysis revealed that this marker could be a predictive marker for chemotherapy response. Median survival time was longer in patients who tested negative for WFA$^+$-M2BP than in patients who tested positive for WFA$^+$-M2BP (371.5 days vs 250.0 days, log-rank test: $P = 0.035$; Fig. 2).

Serum WFA$^+$-M2BP was produced in MDA-PATC53 cells as a consequence of elevated expression of glycosylation enzymes.

To elucidate the production source, we measured WFA$^+$-M2BP levels in cell lysate and supernatant from 4 PDAC cell lines, HEK293T cell line, and an immortalized human pancreatic stellate cell line (hPSC21-S/T cells). In this study, we found that only the MDA-PATC53 cell line originated from PDAC liver metastasis produced WFA$^+$-M2BP independent of cellular M2BP production (Table 6 and Fig. 3).

Using in vitro experiments, we conducted cell proliferation, cell migration, and cell invasion assays to clarify the biological effect of WFA$^+$-M2BP in 3 pancreatic cancer cell lines (Fig. 4). The results showed that WFA$^+$-M2BP did not influence tumor behavior, suggesting that WFA$^+$-M2BP was elevated as a consequence rather than as a cause of tumor progression. The cDNA microarray focused on glycosylation enzymes revealed that several glycosylation enzymes with pro-oncologic function, N-acetylgalactosaminyltransferase-3 and -14 (GALNT-3 and GALNT-14), were upregulated while an anti-oncologic enzyme, neuraminidase 1 (NEU1), was downregulated in MDA-PATC53 compared to Panc-1 (Fig. 5).

4. Discussion

To the best of our knowledge, this was the first study to demonstrate the clinical significance of serum WFA$^+$-M2BP in PDAC. The serum level of WFA$^+$-M2BP was found to be a reliable diagnostic marker of PDAC and predictive marker of metastasis. Moreover, high WFA$^+$-M2BP was closely related to poor prognosis in patients who underwent chemotherapy. The results of in vitro experiments suggested that elevated WFA$^+$-M2BP might be a consequence of altered glycosylation enzymes rather than the cause of disease progression since MDA-PATC53 supernatant with

Table 1
Clinical Characteristics of patients with chronic pancreatitis and pancreatic ductal adenocarcinoma.

	CP (n = 16)	PDAC (n = 24)	P-value
Sex, female/male	1/15	10/14	0.02
Age, years, median (range)	63 (43–83)	65 (46–80)	0.83
Morphology			
Pancreatic duct size, mm, median (range)	4.7 (1.4–13.5)	4.5 (1.0–8.4)	0.87
Pancreatic stone or calcification	68.8% (11/16)	8.3% (2/24)	0.0001
Biliary obstruction	18.8% (3/16)	45.8% (11/24)	0.10
Pancreatic atrophy	50% (8/16)	62.5% (15/24)	0.52

CP: chronic pancreatitis, PDAC: pancreatic ductal adenocarcinoma.
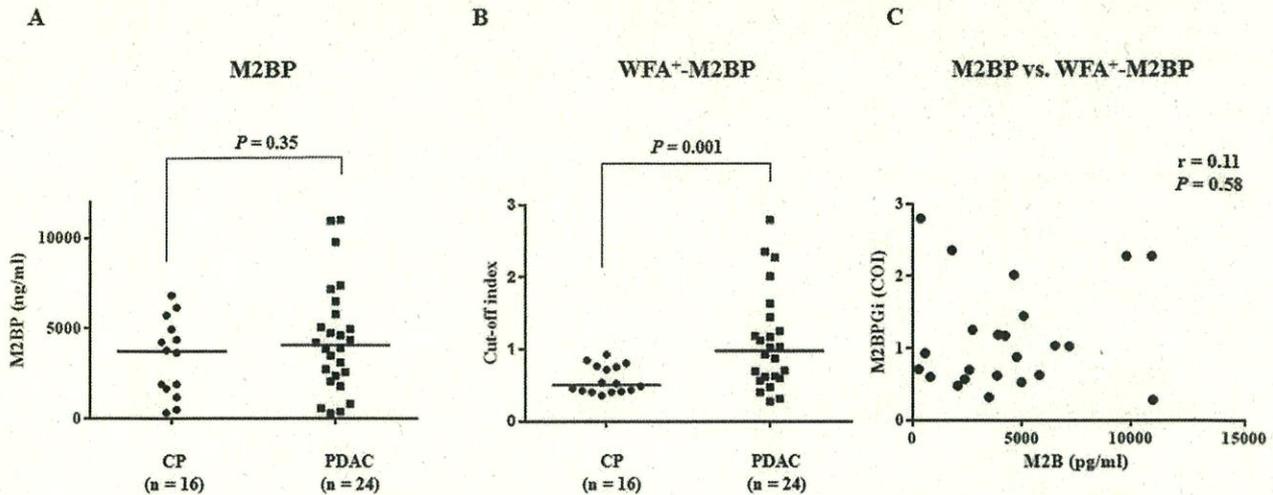
A **M2BP**

B **WFA⁺-M2BP**

C **M2BP vs. WFA⁺-M2BP**

Fig. 1. Serum level of M2BP and *Wisteria floribunda* agglutinin-positive Mac-2 binding protein (WFA⁺-M2BP. (A) (B) The serum level of WFA⁺-M2BP in patients with pancreatic ductal adenocarcinoma (PDAC) was higher than that of in patients with chronic pancreatitis (CP). (C) The serum level of WFA⁺-M2BP was independent of M2BP in correlation analysis (r = 0.11, P = 0.58).

Table 2
Differences in serum markers between chronic pancreatitis and pancreatic ductal adenocarcinoma.

	CP (n = 16)	PDAC (n = 24)	P-value
CEA (ng/ml)	2.85 (0.9–8.7)	3.05 (0.5–29.0)	0.30
CA19-9 (U/ml)	15.5 (0.1–84.5)	344.4 (2.0–22700.0)	<0.001
M2BP (ng/ml)	3732 (323.3–6845.0)	4072 (287.0–11001)	0.35
WFA⁺-M2BP (COI)	0.51 (0.36–0.93)	0.98 (0.28–2.81)	0.001

CP: chronic pancreatitis, PDAC: pancreatic ductal adenocarcinoma, CEA: carcinoembryonic antigen, CA19-9: cancer antigen 19-9, M2BP: Mac-2 binding protein, WFA⁺-M2BP: *Wisteria floribunda* agglutinin-positive M2BP.

WFA⁺-M2BP did not influence tumor behavior.

Since WFA⁺-M2BP was closely related to liver fibrosis, we speculated that pancreatic stellate cells which played a pivotal role in pancreatic fibrosis might produce it [11–13]. However, M2BP is a 90 kDa protein than binds to its receptor, galectin-3, and plays a

role in the regulation of immune response and cell adhesive ability in patients with cancer and infectious diseases [17]. In some cancers, M2BP has been reported to be a useful marker to distinguish cancers from normal controls [17,18]. Since galectin-3 is frequently expressed in PDAC and enhances cell migration and invasion ability, we speculated that serum levels of M2BP could be greater in PDAC than in controls and predict disease progression [19,20]. However, contrary to our speculation, we found that the serum level of M2BP could not be used to distinguish PDAC from CP. Moreover, the serum level of M2BP between metastatic PDAC and non-metastatic PDAC was not significantly different (4249 ng/ml and 3912 ng/ml, P = 0.65). Consequently, we found that the serum level of M2BP was not a useful biomarker for the diagnosis of PDAC and the prediction of disease progression.

On the other hand, our results demonstrated that glycocylated-M2BP, WFA⁺-M2BP, showed significant effect on both diagnosis of cancer and prediction of disease progression. Since the value of

Table 3
Diagnostic yield of CA19-9 and WFA⁺-M2BP in distinguishing chronic pancreatitis and pancreatic ductal adenocarcinoma.

	Sensitivity	Specificity	PPV	NPV	AUC
CA19-9 > 59.2 U/ml	79.2 (57.8–92.9)	93.8 (69.8–99.8)	95.0 (81.7–99.1)	75.0 (61.7–79.1)	85.1 (73.2–97.0)
WFA⁺-M2BP > 1.0 COI	50.0 (29.1–70.9)	100.0 (79.4–100.0)	100 (79.9–100)	57.1 (48.5–57.1)	76.0 (61.3–91.2)

CA19-9: cancer antigen 19-9, WFA⁺-M2BP: *Wisteria floribunda* agglutinin-positive M2BP, PPV: positive predictive value, NPV: negative predictive value, AUC: area under curve.

Table 4
Clinical characteristics between M2BPGi positive and negative results from patients with pancreatic ductal adenocarcinoma.

	WFA⁺-M2BP (+) (n = 12)	WFA⁺-M2BP (-) (n = 12)	P-value
Age	64.5 (55–76)	65 (46–80)	0.92
Tumor size (mm)	29.0 (15.0–60.0)	28.5 (10.0–50.0)	0.85
Sex (M/F)	5/7	9/3	0.21
Location (Ph vs Pb/t)	7/5	8/4	1.00
Stage (UICC) I-III/IV	1/11	7/5	0.009
CEA (ng/ml)	3.4 (1.7–29.0)	3.05 (0.5–24.8)	0.56
CA19-9 (U/ml)	1020 (9.7–4949)	325.5 (2.0–22,700)	0.83
M2BP (ng/ml)	4633 (380.8–10958)	3040 (287.0–11001)	0.18

M2BP: Mac-2 binding protein, WFA⁺-M2BP: *Wisteria floribunda* agglutinin-positive M2BP, CEA: carcinoembryonic antigen, CA19-9: cancer antigen 19-9.

Table 5
Diagnostic yield of CA19-9 and WFA + -M2BP in detecting metastasis.

	Sensitivity	Specificity	PPV	NPV	AUC
CA19-9 > 291.5 U/ml	62.5	73.3	78.6	55.6	66.7
	(24.5–91.5)	(44.9–92.2)	(63.1–90.4)	(31.5–74.0)	(44.1–89.2)
WFA+-M2BP > 1.0 COI	68.8	87.5	90.9	58.3	75.0
	(54.8–73.8)	(59.6–97.7)	(70.8–98.3)	(39.9–65.1)	(56.4–81.8)

CA19-9: cancer antigen 19-9, WFA+-M2BP: Wisteria floribunda agglutinin-positive M2BP, PPV: positive predictive value, NPV: negative predictive value, AUC: area under curve.
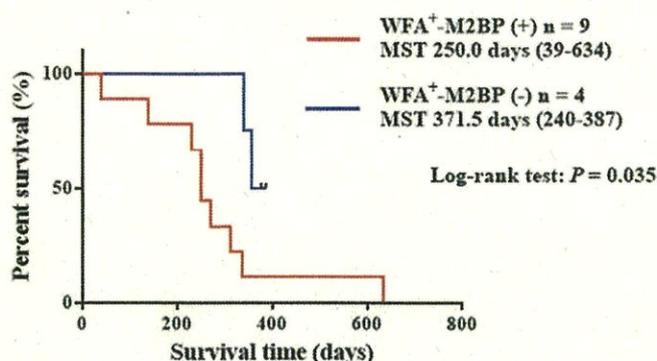

Fig. 2. Results of survival analysis in patients who underwent chemotherapy. Median survival time was longer in WFA+-M2BP negative patients (n = 4) than in WFA+-M2BP positive patients (n = 9) (371.5 days vs. 250.0 days, P = 0.035).

Table 6
Differences in WFA+-M2BP production in cell lines.

Cell line	WFA+-M2BP		Origin of cell
	Cell lysate	Supernatant	
HEK293T	−	−	Normal kidney
Panc-1	−	−	Primary PDAC
MiaPaca-2	−	−	Primary PDAC
BxPC-3	−	−	Primary PDAC
MDA-PATC 53	+	+	Liver metastasis PDAC
hPSC-S/T21	−	−	Pancreatic cancer tissue

WFA+-M2BP: Wisteria floribunda agglutinin-positive Mac-2 binding protein, PDAC: pancreatic ductal adenocarcinoma, hPSC-S/T: human pancreatic stellate cell.
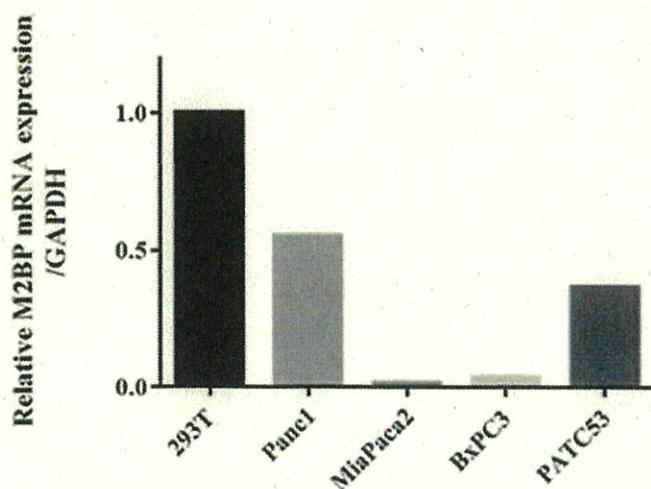
Fig. 3. Relative M2BP mRNA expression in each cell line.

serum WFA+-M2BP was not related to the level of M2BP in PDAC, glycosylation could be a strong factor that affected protein function. Glycosylation is a common protein post-translational modification that can be altered in various physiological conditions and diseases. Aberrant glycosylation has been observed to be a molecular feature of malignant transformation in various cancers [21,22]. Recently, Pan et al. observed aberrant N-glycosylation levels of mucin-5AC (MUC5AC), carcinoembryonic antigen-related cell adhesion molecule 5 (CEACAM5), insulin-like growth factor-binding protein (IGFBP3), and galectin-3-binding protein (LGALS3BP, which is equivalent to M2BP) in pancreatic cancer tissue [22]. Because Wisteria floribunda agglutinin is thought to recognize the GalNAc residue of N-glycans and O-glycans or the clustered LacNAc (Gal-GlcNAc) structure, it might be associated with N-glycosylation on M2BP [23]. The cDNA microarray focused on glycosylation enzymes revealed that N-acetylgalactosaminyltransferase-3 and -14 (GALNT-3 and GALNT-14) were upregulated while NEU1 was downregulated in MDA-PATC53 compared to Panc-1. GALNT enzymes belong to a large subfamily of glycosyltransferases residing in the Golgi apparatus. GALNT enzymes catalyze the first step in the O-glycosylation of mammalian proteins by transferring GalNAc to peptide substrates. Among them, GALNT-3 and GALNT-14 have been associated with cancer progression [24,25]. NEU1 is a major endogenous sialidase in human cells and forms a multi-enzyme complex including protective protein and β-galactosidase in lysosomes. It is proven to be an anti-oncologic factor [26]. Our data reinforced these authors' deductions about the role of the glycosylation of M2BP in pancreatic tumorigenesis. Regarding CP, the aberrant glycosylation pattern likely differs from that of PDAC, as previously described [22,27].

Despite the potential benefit of glycosylation markers in cancer diagnosis, they have not been widely utilized in the clinical setting, because of lack of commercially available equipment that would enable us to detect the markers automatically. A recently developed, fully automated system using a chemiluminescence enzyme immunoassay machine (HISCL-2000i; Sysmex, Kobe, Japan) is now widely available in daily clinical practice, mainly for the assessment of liver cirrhosis. Previous studies revealed its clinical impact on assessment of liver fibrosis and prediction of hepatocellular carcinoma. Even though the mechanism of elevated serum WFA+-M2BP has not been elucidated and its biological function is still unknown, the present study is the first to reveal that WFA+-M2BP is produced by PDAC cells with altered glycolytic enzymes that could influence tumor progression.

One limitation of our study is the relatively small number of samples that were collected at a single institution. These results should be evaluated in future studies that focus on clarifying the molecular events that may occur after the glycosylation of M2BP and affect cancer behavior.

In conclusion, serum WFA+-M2BP might be a useful biomarker in the diagnosis of PDAC and the assessment of disease progression. Greater understanding of the biological role of aberrant glycosylation in pancreatic disease will help us to develop novel diagnostic
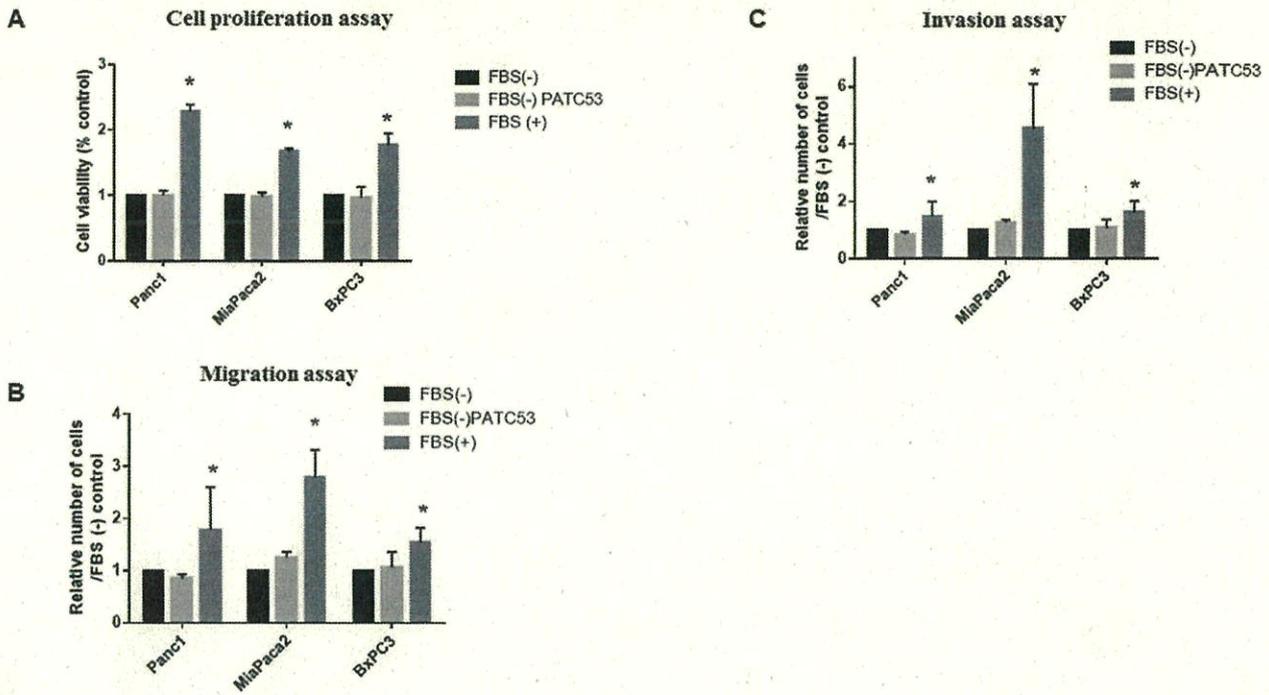
Fig. 4. Results of cell proliferation assay, cell migration, and cell invasion assay (Fig. 4A–C). Data are expressed as means ± s.e.m. *P < 0.05 vs. no fetal bovine serum control. FBS (-): RPMI-1640 media without any FBS, FBS (+): RPMI-1640 media with 10% FBS, FBS (-) PATC53: MDA-PATC53 conditioned supernatant without FBS.
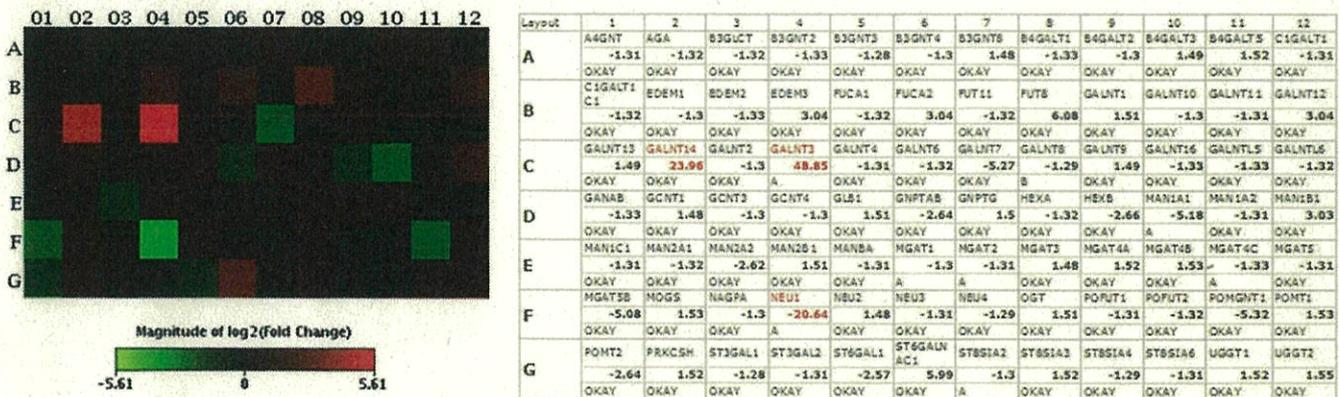

Fig. 5. Results of the cDNA microarray. Relative gene expression of MDA-PATC53 compare to Panc-1 was analyzed. Pro-oncologic function protein, N-acetylgalactosaminyl-transferase-3 and -14 (GALNT-3 and GALNT-14), were upregulated while an anti-oncologic enzyme, neuraminidase 1 (NEU1), was downregulated.

methods and treatments for PDAC.

Acknowledgements

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