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High Expression of Karyopherin-α2 and Stathmin 1 is Associated With Proliferation Potency and Transformation in the Bile Duct and Gall Bladder Epithelia in the Cases of Pancreaticobiliary Maljunction

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Backgrounds and Objectives: Pancreaticobiliary maljunction (PBM) may be associated with an increased frequency of gall bladder cancer with no bile duct dilation. Karyopherin-α2 (KPNA2) and stathmin 1 (STMN1) were reported to play important roles in carcinogenesis and cancer progression.

Methods: Fifteen patients with PBM who underwent surgical resection between 1999 and 2014 were included in this study. Using immunohistochemistry, we investigated the expression of p53, Ki-67, KPNA2, and STMN1 in normal biliary tract epithelium, hyperplastic epithelium, and cholangiocarcinoma (CC) tissues.

Results: Nuclear expression of KPNA2, p53, and Ki-67 expression was detected in hyperplastic epithelium and CC tissues. High KPNA2 expression was significantly associated with gender ($P = 0.04$), p53 nuclear accumulation ($P = 0.00435$), and Ki-67 expression ($P = 0.0443$) in the gall bladder and bile duct of PBM. On the other hand, STMN1 was only expressed in CC tissues and was not observed in normal bile duct and hyperplastic epithelia.

Conclusions: KPNA2 might be a useful marker of hyperplasia, dysplasia, and carcinogenicity in patients with PBM. STMN1 evaluation might be a cancer-specific marker for CC patients with PBM similar as that for other cancers.

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KEY WORDS: **inportin; stathmin 1; Op18; biliary tract cancer; PBM**

INTRODUCTION

Pancreaticobiliary maljunction (PBM) is an innate anomaly in which the pancreatic and bile ducts meet outside the duodenal wall [1,2]. Because of the incompetent union at the sphincter muscle of Oddi, this anomaly causes regurgitation of bile and pancreatic juice. PBM has been associated with an increased frequency of gall bladder cancer with no bile duct dilation [3]. With the continued stimulation of chronic inflammation, the gall bladder mucosa may change into a hyperplastic epithelium. The hyperplasia–dysplasia-carcinoma sequence has been reported as a mechanism of biliary carcinogenesis in PBM [4,5]. Studies have reported that p53 and Ki-67 expression was altered and dependent on the progression of the sequence [6,7]. Because cholangiocarcinomas (CC) often undergo local progress permeation infiltration and metastases, radical excision may be difficult; therefore, it has a poor prognosis [8,9]. It is important to develop a diagnostic biomarker to predict lethal CC progression in patients with PBM. Moreover, such a marker might be a promising molecular candidate for targeting carcinogenesis in the biliary systems of patients with PBM.

Stathmin 1 (STMN1) is one of the cytosolic phosphoproteins that regulates microtubule dynamics by promoting microtubule destabilization [10]; therefore, it is a microtubule destabilizer. STMN1 plays an important role in carcinogenesis and cancer progression [11]. Many types of human malignancies have expressed higher STMN1 levels than non-cancerous tissues, and this expression has been associated with cancer progression and therapeutic resistance, particularly against microtubule agents. In addition, STMN1 is also known as oncoprotein 18 (op18). Previously, many researchers have reported the clinical significance of STMN1 expression in malignant tumors, including breast cancer [12,13], prostate cancer [14], cervical cancer [15], malignant mesothelioma [16], gastric cancer [17,18], hepatocellular carcinoma [19], oral squamous cell carcinoma, endometrial cancer [20], colorectal cancer [21], and upper urinary

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tract urothelial carcinoma [22]. In particular, we first presented the clinical significance of STMN1 in sporadic CC [23]. These studies suggested that STMN1 could be a potential target for diagnosis and treatment even in CC with PBM; however, the exact significance of STMN1 in biliary systems, including those with CC and PBM, has not yet been determined.

Recent studies have identified high expression levels of karyopherin-α2 (KPNA2) as a marker of poor prognosis in a variety of cancer types [24], including breast cancer [25], malignant melanoma [26], esophageal cancer [27], lung cancer [28], ovarian cancer [29], prostate cancer [30], brain cancer [31], and hepatocellular carcinoma [32]. KPNA2 has been reported to transport several macromolecules larger than 50 KDa or other complex proteins from the cytoplasm into the nucleus. The cargo proteins transported by KPNA2 include several cancer-related proteins, including p53, myc, E2F, MRN complex, and the STAT family. Among these proteins, p53, myc, STAT, and the MRN complex have been suggested to play important roles in carcinogenesis, proliferation, and the resistance to chemotherapy in bile duct cancer [33–36]. However, few previous studies have addressed whether KPNA2 expression is related to the progression to hyperplasia and carcinogenesis in the bile duct and gall bladder of patients with PBM.

The purpose of this research was to clarify the significance of KPNA2 and STMN1 expression in the biliary tracts of patients with PBM, including the normal biliary epithelium, hyperplastic epithelium, and cancerous tissue using immunohistochemistry. Moreover, we examined the changes in the expression of KPNA2 and STMN1, the proliferation marker Ki-67, and transformation-related marker p53 in the hyperplasia–dysplasia-carcinoma sequence of these samples.

MATERIALS AND METHODS

Patients and Samples

Fifteen patients with PBM who underwent surgical resection in our department between 1999 and 2014 were included in this study. There were four males and 11 females. The mean age of patients was 48.5 years (range, 16–77). There were four patients with PBM who had cancer of the gall bladder (two cases) or bile duct (two cases). The Todani classification of the PBM cases were graded as type Ia (n = 3), type Ic (n = 3), type IVa (n = 4), type V (n = 1), and non-dilatation (n = 4), according to a previous report [3]. None of patients had received neoadjuvant chemotherapy and/or irradiation before the surgical resection. Written informed consent was obtained from all patients. This study was approved by the institutional review board at Gunma University Hospital.

Immunohistochemical Staining

A paraffin-embedded block for each of the PBM specimens was cut into 2 μm-thick sections and mounted on glass slides. Each section was deparaffinized using xylene and dehydrated in alcohol. Endogenous peroxidase was inhibited using 0.3% H_2O_2/methanol for 30 min at room temperature. The sections were soaked in heated water with 0.5% Immunosaver (Nishin EM, Tokyo, Japan) at 98°C for 45 min. Non-specific antigens were blocked by Protein Block Serum-Free (DAKO, Glostrup, Denmark) at room temperature for 30 min. The sections were then incubated with primary antibodies against KPNA2 (Abcam, Tokyo, Japan, Rabbit anti-KPNA2 polyclonal antibody, 1: 400), STMN1 (Santa Cruz Biotechnology, Santa Cruz, CA, Mouse monoclonal anti-STMN1 antibody, 1: 100), p53 (DAKO, DO-7, Monoclonal Mouse Anti-Human p53, 1:100), and Ki67 (DAKO, MIB-1, Monoclonal Mouse Anti-Human Ki-67, 1: 200) for 24 hr at 4°C. After washing with phosphate buffered saline, the Histofine Simple Stain MAX-PO (MULTI) kit (Nichirei, Tokyo, Japan) was applied for visualizing the primary antibody and incubated for 45 min. The chromogen 3,3′-diaminobenzidine tetrahydrochloride was applied as a 0.02% solution containing 0.005% H_2O_2 in 50 mM of ammonium acetate–citrate acid buffer (pH 6.0). Finally, counterstaining of the nucleus was performed using Mayer's Hematoxylin solution. We adopted a negative control by replacing the primary antibody with phosphate buffered saline in 0.1% bovine serum albumin and confirmed no detectable staining with the negative control.

Assessment of KPNA2, STMN1, p53, and Ki-67 Expression

We evaluated the cytoplasmic staining of STMN1 in the normal epithelium, hyperplastic epithelium, and cancerous tissue of patients with PBM. KPNA2-positive cells were defined as those with a brown-stained nucleus, regardless of staining intensity. Three staining patterns were identified: positive, positive cells in most of the lesion; moderate, positive cells aggregated in a focal area of the lesion and small numbers of isolated positive cells scattered throughout the lesion; and negative. Cytoplasmic STMN1 was scored as follows: 0, no staining; 1+, 1–10%; 2+, 11–50%; and 3+, 51–100%. The optimal cut-off point was defined as follows: grades 0, 1, and 2 were considered negative, and grade 3 was designated as positive. p53-positive cells were defined as those with a brown-stained nucleus, regardless of the staining intensity. The following four staining patterns were identified: positive cells in most of the lesion (diffuse), positive cells aggregated in a focal area of the lesion (nested), small numbers of isolated positive cells scattered throughout the lesion (scattered), and negative. Positive p53 protein expression was defined as either a diffuse or nested pattern and negative p53 protein expression was defined as a scattered pattern throughout the lesion or negative as previously described [37,38]. For the evaluation of Ki-67 expression, a positive cell frequency of more than 10% was assessed as positive.

Statistical Analysis

Data for continuous variables were expressed as mean ± standard deviation (SD). Associations between KPNA2 expression and clinicopathological characteristics were analyzed using the chi-square and Mann–Whitney U tests. All differences were considered statistically significant at $P < 0.05$. All statistical analyses were performed using the JMP software package (SAS Institute Inc., Cary, NC).

RESULTS

Expression Analysis of KPNA2, STMN1, p53, and Ki-67 in the Gall Bladder and Bile Duct of Patients With PBM by Immunohistochemistry

Immunohistochemistry analysis showed that KPNA2 was expressed in cell nuclei. Therefore, nuclear KPNA2 expression was evaluated in the gall bladder or bile duct of 15 patients with PBM. In all cases, nuclear KPNA2 expression was not observed in normal gall bladder or bile duct epithelium (Fig. 1A) and (Table I). However, the expression level of KPNA2 increased during hyperplasia (66.7%, 10/15) and in cancerous tissues (100%, 5/5) (Fig. 1A) and (Table I). We investigated the correlation of KPNA2, p53, and Ki-67 expressions by immunohistochemistry in representative sections of normal, hyperplastic, and cancerous tissues from identical cases. High KPNA2 expression sections demonstrated the enhanced expression of p53 and Ki-67 in hyperplastic and cancerous tissues (Fig. 1B,C).

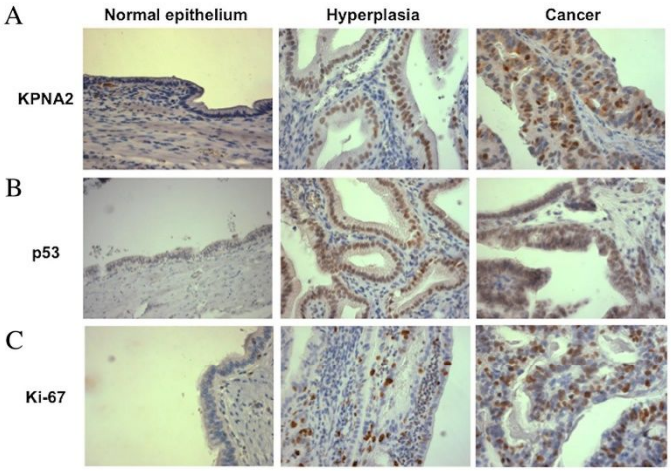

Fig. 1. KPNA2, p53, and Ki-67 expression in representative tissues of patients with PBM. (**A**) The expression of nuclear KPNA2 in representative normal epithelium, hyperplastic tissue, and cancerous tissue in the biliary tract of patients with PBM (original magnification: ×20, ×40, and ×40, respectively). (**B**) The expression of nuclear p53 in representative normal epithelium, hyperplastic tissue, and cancerous tissue in the biliary tract of patients with PBM (original magnification: ×20, ×40, and ×40, respectively). (**C**) The expression of nuclear Ki-67 in representative normal epithelium, hyperplastic tissue, and cancerous tissue in the biliary tract of patients with PBM (original magnification: ×20, ×40, and ×40, respectively).

STMN1 was expressed only in the CC tissues and was not observed in the normal bile duct or hyperplastic epithelium in all cases (Fig. 2A). STMN1 and p53 expression level in CC tissues of patients with PBM was 80%. STMN1 and p53 expression in CC tissues had the same frequency (Table I). p53 expression level in the hyperplastic epithelium of patients with PBM was 40%. Also, p53 expression was not observed in the normal epithelium (Fig. 2B) and (Table I). Furthermore, Ki-67 expression level in the hyperplastic epithelium of patients with PBM was 26.7% (Fig. 2C) and (Table II). p53 and Ki-67 expression was induced depending on the progression of the hyperplasia-carcinoma sequence in patient with PBM.

Relationships Between the Expression of KPNA2 and Clinicopathological Factors in the Biliary Tract of Patients With PBM

We divided our 15 samples into three groups according to the intensity of nuclear KPNA2 staining in the gall bladder or bile duct with hyperplasia. The correlations between KPNA2, p53, and Ki-67 expressions and clinicopathological characteristics are shown in Table II. High KPNA2 expression was significantly associated with gender ($P = 0.04$), p53 nuclear accumulation ($P = 0.0435$), and Ki-67 expression ($P = 0.0443$). However, there were no significant differences with respect to age, dilatation, or cancer coexistence.

TABLE I. p53, Ki67, KPNA2, and STMN1 Expression in the Biliary System of CC Tissues of Patients

	Normal biliary epithelium* (two cases without normal biliary epithelium)	Hyperplasia (15 PBM cases)	Cancers (five lesions from four cancer cases with PBM)
p53	0% (0/13)	40% (6/15)	80% (4/5)
Ki-67	0% (0/13)	26.7% (4/15)	80% (4/5)
KPNA2	0% (0/13)	66.7% (10/15)	100% (5/5)
STMN1	0% (0/13)	0% (0/15)	80% (4/5)

*Two cases without nornal biliary epithelium.

Analysis of STMN1 Expression in CC Tissues of Patients With PBM Using Immunohistochemistry

STMN1 was highly expressed in the cytoplasm of the cancerous tissue and not expressed in the cytoplasm of the normal or hyperplastic epithelium (Fig. 3A,B). In addition, STMN1 was not expressed in the adenoma tissue. On the other hand, STMN1 was expressed in the carcinoma in situ (Fig. 3C). STMN1 was expressed only in the cancerous tissue. In particular, CC cells in the invasion front were expressing high levels of STMN1 compared with that expressed by the main CC tumors (Fig. 3D).

DISCUSSION

In this study, we clarified that STMN1 expression was higher in only the CC lesions derived from patients with PBM and that KPNA2, Ki67, and p53 expression was induced in the hyperplastic epithelium and cancerous tissues of patients with PMB depending on the progression of the hyperplasia-carcinoma sequence.

Progression from a hyperplastic state to a cancer lesion in the biliary tract is important and represents a lethal problem in patients with PBM, including many young individuals because of a congenital anatomical anomaly. The overexpression of p53 is reported to be associated with carcinogenesis in the mucosal epithelia of the biliary system in patients with PBM [39]. The expression rate of p53 in cancer, carcinoma in situ, and dysplasia was 65%, 44.7%, and 2.4%, respectively [40]. In this

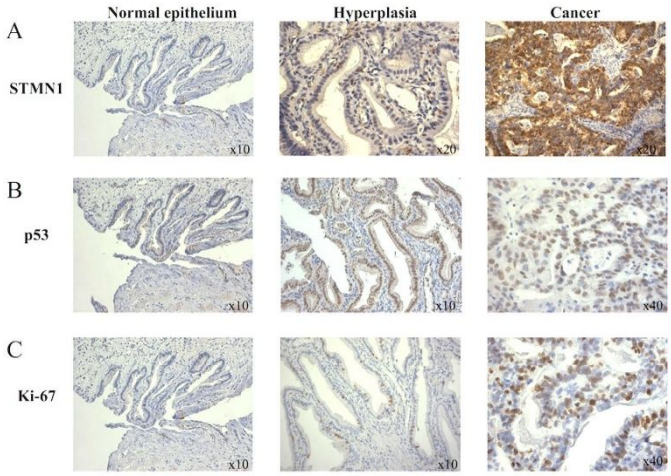

Fig. 2. STMN1, p53, and Ki-67 expression in representative tissues of patients with PBM. (**A**) Cytoplasmic STMN1 expression in the representative normal epithelium, hyperplastic epithelium, and cancerous tissues of the biliary tract of patients with PMB (original magnification: ×10, ×20, and ×20, respectively). (**B**) Nuclear p53 expression in the representative normal epithelium, hyperplastic epithelium, and cancerous tissues of the biliary tract of patients with PMB (original magnification: ×10, ×10, and ×40, respectively). (**C**) Nuclear Ki-67 expression in the representative normal epithelium, hyperplastic epithelium, and cancerous tissues of the biliary tract of patients with PMB (original magnification: ×10, ×10, and ×40, respectively).

study, the positive detection rate for p53 was 40% and 80% for hyperplasia and cancers, respectively. This positive rate is slightly higher than that cited in previous reports [40]. The nuclear KPNA2 accumulation in the biliary tract mucosa of patients with PMB was significantly related to the expression of transformation-related protein p53 and the proliferation marker Ki-67, and such accumulation was detected in all cancerous tissues (100%, 5/5 cancer lesions from four patients with PBM). KPNA2 might overcome the existing hyperplasia-carcinogenesis marker p53 in patients with PBM.

TABLE II. Relationship Between the Expression of KPNA2, p53, and Ki-67 and Clinicopathological Factors in the Biliary Tract of PBM Patients

	KPNA2 expression in hyperplasia of PBM			
Factor	Negative, n = 5	Moderate, n = 3	Positive, n = 7	*P*-value
Age (yr) (mean ± SD)	55.6 + 12.9	22.7 + 9.86	54.6 + 18.8	0.0728
Gender				
Male	0	0	4	0.04*
Female	5	3	3	
p53 accumulation				
Absent	5	2	2	0.0435*
Present	0	1	5	
Ki-67 expression				
Low <10%	5	3	3	0.0443*
High >10%	0	0	4	
Dilatatoin				
Absent	2	0	2	0.46
Present	3	3	5	
Cancer coexistence				
Absent	4	3	3	0.51
Gall bladder	0	0	2	
Bile duct	1	0	1	
Pancres	0	0	1	

*$P < 0.05$.

Biliary carcinogenesis secondary to chronic inflammation has been a clinically significant problem, and novel markers for diagnosis are required. In this study, STMN1 was not expressed in non-cancerous portions of the tissue and in the hyperplastic epithelium and was very highly expressed in the cancerous part. STMN1 has been reported to be expressed in higher levels in various cancers compared with those normal tissues, and has been associated with poor prognosis, cancer progression, and taxane anticancer agent resistance in idiopathic CC [23]. These data were consistent with previous studies from many researchers. STMN1 might be a promising candidate for targeted therapy and for controlling the progression of refractory CCs with or without PBM.

In a preclinical study, the measurement of KPNA2 and STMN1 levels in the serum of cancer patients by enzyme-linked immunosorbent assay suggested that a high expression of these proteins represents a marker for lung and urinary cancer [28,41,42]. From these observations, KPNA2 and SMTN1 testing with liquid samples from preoperative or prediagnostic patients with PBM might represent a new marker to predict carcinogenesis in the biliary tract of patients with PBM.

Several factors have been reported as STMN1 regulators, including p53, p27, and the PI3K/AKT pathway [43,44]. These factors may be related to the activation of mitogen-activated protein kinase (MAPKs) and signal transducer and activator of transcription 3 (STAT3) in CC [45–47]. From these data, it was suggested that STMN1 regulation by these factor might be important in CC carcinogenesis in part via activation of STAT3 and MAPKs. On the other hand, STMN1 expression is induced by p53 mutation [48]. STMN1 and p53 expression in CC patients with PBM was 80%. STMN1 and p53 expression had the same frequency. However, STMN1 expression was not detected in the hyperplastic epithelium with p53 accumulation;

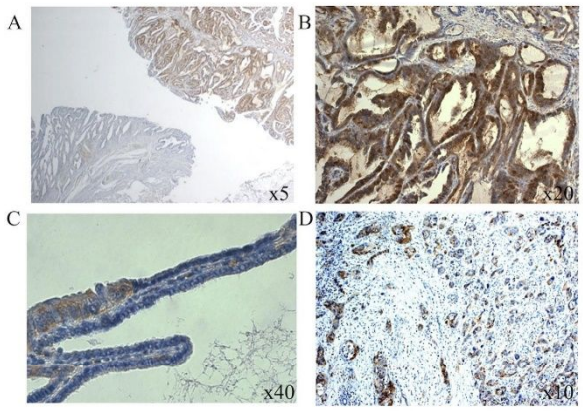

Fig. 3. Immunohistochemical staining of stathmin 1 (STMN1) in cholangiocarcinoma (CC) tissues of patients with PBM. (**A**) STMN1 expression in hyperplastic epithelium (left side) and CC tissues (right side) (original magnification ×5). TMN1 was highly expressed in the cytoplasm of the cancerous tissue. STMN1 was not expressed in the cytoplasm of the normal epithelium and hyperplastic mucosa. (**B**) A representative imaging of a case of high STMN1 expression in CC tissue (original magnification ×20). Part B is a magnified image of Part A. (**C**) STMN1 expression in the marginal region between carcinoma in situ and the adjacent non-cancerous tissue (original magnification ×40). STMN1 was not expressed in the adenoma. On the other hand, STMN1 was expressed in the carcinoma in situ tissue. (**D**) STMN1 expression in the CC tissues between invasion line (left side) and the main tumors (right side) (original magnification ×10). CC cells in invasion line expressed high levels of STMN1 compared with the main CC tumors.

therefore, p53 may not be the main adjustment mechanism for STMN1 in CC patients with PBM.

Importin α proteins, including KPNA2, have many important cellular functions in both cancerous and non-cancerous tissues because fundamental mechanisms such as proliferation and DNA repair are necessary for all types of cells [49,50]. The main importin α protein in a variety of non-cancerous adult tissues is importin α5 (KPNA1) [51]. However, in the present study, we focused on the cancer-specific importin α family member KPNA2, which is over-expressed in only cancerous and stem cells [49]. KPNA2 expression is induced by the activation of the KPNA2 promoter by E2F [52], which is expressed in several cancerous and stem cells [53,54]. E2F is reported to be one of the KPNA2 cargo proteins, and the nuclear translocation of E2F by KPNA2 is needed to activate transcriptional capability. In this study, it is suggested that a positive feedback loop involving KPNA2 and E2F is an important regulatory mechanism for KPNA2 in the biliary system and cancers in patients with PBM.

Therefore, KPNA2 expression in the biliary tract mucosa of patients with PBM was correlated to the expression of p53 and Ki67. KPNA2 is therefore expected to be a useful marker of hyperplasia, dysplasia, and carcinogenicity. STMN1 expression contributed to carcinogenesis in the biliary systems of patients with PBM. STMN1 evaluation might be a cancer-specific marker for CC patients with PBM similar as that for other cancers. Moreover, many researchers have been interested in the possibility of KPNA2 and STMN1 as a therapeutic target against refractory cancers. Targeting strategy against these proteins may be a promising molecular target for controlling the progression of CC with PBM.

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