Metadherin overexpression in perihilar cholangiocarcinoma is associated with lymph node metastasis and poor prognosis

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Abstract. Metadherin (MTDH) is a protein that is also named astrocyte elevated gene-1, and is highly expressed in a number of different tumor tissues. Although the expression of MTDH is associated with tumor invasion and recurrence, the expression of this protein in perihilar cholangiocarcinoma (PCCA) and its clinical use have not yet been investigated. In the present study, the expression of MTDH in patients with PCCA was investigated in order to determine its clinicopathological use. An immunohistochemical method was used to detect MTDH expression and the epithelial-mesenchymal transition markers E-cadherin and vimentin in 66 cases of PCCA. In addition to the expression of MTDH, the clinical and pathological data and the postoperative outcomes were analyzed. The MTDH positive expression rate was 48.5% (32/66) in PCCA. A significantly higher MTDH expression level was identified in the poor tumor differentiation group compared with the well differentiation group (P=0.007). In the positive lymph node metastasis group, a significantly higher MTDH expression level was revealed compared with the negative lymph node metastasis group (P=0.023). No association was noted with regard to the expression of MTDH and the variables age, sex, tumor diameter, tumor grade and tumor classification stage. Positive MTDH expression was significantly associated with high vimentin expression (P=0.037) compared with negative vimentin expression and inversely associated with positive E-cadherin expression compared with negative E-cadherin expression (P=0.030). Survival analysis suggested that the high MTDH expression group was associated with a worse overall survival (OS) rate and recurrence free survival (RFS) rate compared with the low MTDH expression group (P<0.001 and P=0.01, respectively). Cox regression analysis indicated that the Tumor-Node-Metastasis, surgery margin and high MTDH expression were independent OS and RFS factors for PCCA. MTDH expression may serve an important function in PCCA tumor growth and metastasis. Targeting MTDH may have important therapeutic applications for patients with PCCA.

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Introduction

Perihilar cholangiocarcinoma (PCCA), also called Klatskin tumor, is a rare disease that has a poor prognosis (1). PCCA presents in the left and right bile duct bifurcation, accounting for 40-60% of bile duct carcinomas and 58-75% of extrahepatic bile duct carcinomas (2). The prognosis of PCCA is associated with the histopathological results of the surgical tumor edge, and the tumor histological grading and staging are all associated with post-operative morbidity and tumor lymph node invasion (3). Complete tumor resection remains the only effective treatment method for PCCA and additional adjuvant treatment is currently absent. However, only 30% of patients with PCCA are able to achieve complete surgical resection (4). In previous years, molecular targeting therapy has achieved satisfactory results in certain cases (5). Therefore, the investigation of the pathogenesis of PCCA is imperative for the identification of novel genes that may be targeted therapeutically.

The metadherin (MTDH) gene is located on chromosome 8 long arm zone 22 (8 q22) and encodes a protein ~64 kDa in size (6). MTDH was originally cloned in human embryonic astrocytes that were infected with human immunodeficiency virus type 1 and was initially named astrocyte increase gene 1 (7). MTDH has been demonstrated to participate in breast cancer metastasis to the lung in a mouse model (7). Consequently, it was identified as a transfer adhesion gene/protein (8). Previously, a number of studies have demonstrated high MTDH expression in liver and breast cancer, osteosarcoma and other malignant tumor types (9-12). In addition, high MTDH expression is associated with a poor prognosis in patients with cancer (13).

However, the expression levels and the clinical significance of MTDH in PCCA have not yet been investigated. In the present study, an immunohistochemical method was adopted to detect MTDH expression in 66 cases of PCCA and the

potential application of MTDH as a prognostic factor of PCCA was examined.

Materials and methods

Study population. The present study was ethically approved by the Medical Ethics Society of Taihe Hospital Affiliated with Hubei University of Medicine (Hubei, China). A total of 66 patients with PCCA received surgical treatment at Taihe Hospital Affiliated with Hubei University of Medicine. The patients provided written informed consent for the use of their tumor specimens in the present study. All surgically resected cholangiocellular carcinoma specimens and non-neoplastic bile ducts exhibited clear pathological diagnosis with haemotoxylin and eosin-stained slides. In brief, the 4 μm-thick sections were deparaffinized and hydrated in 100% alcohol for 5 min and 80% alcohol for 5 min, stained with hematoxylin for 10 min and stained with eosin for 30 sec at room temperature. The slides were observed using a light microscope (magnification, x200). No postoperative complications were observed and therefore it was not further discussed in all included patients. The specimens were fixed with 4% paraformaldehyde for 24 h at room temperature and were paraffin embedded at room temperature. The clinical data including age, sex, tumor size, lymph node metastasis, tumor infiltration depth, histological grade and tumor stage (14) were obtained from each medical record. The recurrence and distant metastasis were assessed by clinical and/or imaging diagnostic methods, which included computer tomography (CT) and magnetic resonance imaging.

Immunohistochemistry. An immunohistochemical staining method was applied in order to detect MTDH expression in PCCA paraffin embedded 4 μm-thick slides. In brief, the slides were deparaffinized and hydrated in a 100% alcohol for 5 min and 80% alcohol for 5 min. Antigen retrieval was performed with citrate buffer at 98˚C for 10 min and endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide in methanol for 15 min at room temperature. An anti-human MTDH rabbit monoclonal antibody (rabbit monoclonal, cat. no. EP4445; 1:100; Abcam, Cambridge, MA, USA) was used to detect MTDH expression. Vimentin (rabbit monoclonal, cat. no. 5741; 1:100; Cell Signaling Technology, Inc., Danvers, MA, USA) and E-cadherin (mouse monoclonal, cat. no. 14472; 1:100; Cell Signaling Technology, Inc.) were used to detect the expression of epithelial-to-mesenchymal (EMT) markers. Samples were incubated with primary antibodies overnight at 4˚C. Subsequently, the samples were incubated with horseradish peroxidase universal IgG secondary antibody (cat. no. sc69786; 1:1,000. Santa Cruz Biotechnology, Inc.) for 30 min at 37˚C. A histostaining kit (cat. no. SP-9001; OriGene Technologies, Inc., Beijing, China) was used to visualize the antibody binding on the slides, according to the manufacturer's protocol. The slides were then counterstained with hematoxylin for 5 min at 37˚C. The slides that contained no primary antibody were used as negative controls and the breast cancer tissues slides that had been already confirmed to overexpress the MTDH protein were used as positive controls. The slides were observed using a light microscope (magnification, x200).

Immunohistochemical evaluation. A semi-quantitative assessment of MTDH expression was performed by measuring the percentage of positive cells, as previously described (8). The staining intensity was scored as 0, negative; 1, weak; 2, moderate; and 3, strong. The percentage of positive cells was scored as 0, negative or <5%; 1, 6-25%; 2, 26-50%; 3, 51-75%; and 4, >76%. The final staining score was calculated by the score of the staining intensity multiplied by the proportion of positively stained cells. A total score of <2 was considered to indicate a low MTDH expression, while a score ≥2 indicated a high MTDH expression. All the sections were assessed by two experienced pathologists, and 3 cases of inconsistent immunohistochemical results were reviewed again by the two pathologists in order to obtain the final pathological diagnosis.

Follow-up. The follow-up was examined by carbohydrate antigen 19-9, ultrasonography or abdominal CT and chest radiography every 3 months for the first 2 years following surgery. Overall survival (OS) rate was calculated from the date of resection to the date of mortality or last follow-up. Recurrence free survival (RFS) rate was calculated between the date of resection to the date of tumor recurrence or the day of mortality or last follow-up.

Statistical analysis. SPSS (version 17.0; SPSS, Inc., Chicago, IL, USA) was used in the present study. The expression of MTDH and the clinical and pathological factors including age, sex, tumor size, capsular invasion, lymph node metastasis, tumor classification stage and distant metastases during diagnosis were analyzed using the χ^2 test or the exact probability analysis (χ^2 test or Fisher's exact test). The OS time was defined as the time from the cancer diagnosis until the patient mortality prior to and during follow-up. The recurrence free survival (RFS) time was defined as the time from the initial PCCA diagnosis to the time point of cancer recurrence prior to and during follow-up. Survival analysis was performed using the Kaplan Meier method and the log-rank test in order to compare the survival differences according to the MTDH expression status. Cox's regression model was used for the survival analysis of multiple pathological factors. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Patient data. The clinical data that were obtained from each medical record are presented in Table I, including age at diagnosis, sex, tumor size, the depth of invasion, histological grade, nodal metastasis and tumor stage according to the American Joint Committee on Cancer (15). The mean age at diagnosis of the disease was 57.4 years (range, 32-78 years). In total, 21 (31.82%) cases were female and 45 (68.18%) were male. Clinical follow-up was available for all patients.

Expression of MTDH in non-neoplastic bile ducts and cholangiocellular carcinoma. MTDH expression was detected by immunohistochemical methods in patients with PCCA. Fig. 1 represents the MTDH expression in tumor specimens and in matched normal tissues. MTDH was negatively and/or weakly expressed in the cytoplasm and in the cell membrane of cholangiocytes derived from normal bile ducts. However,

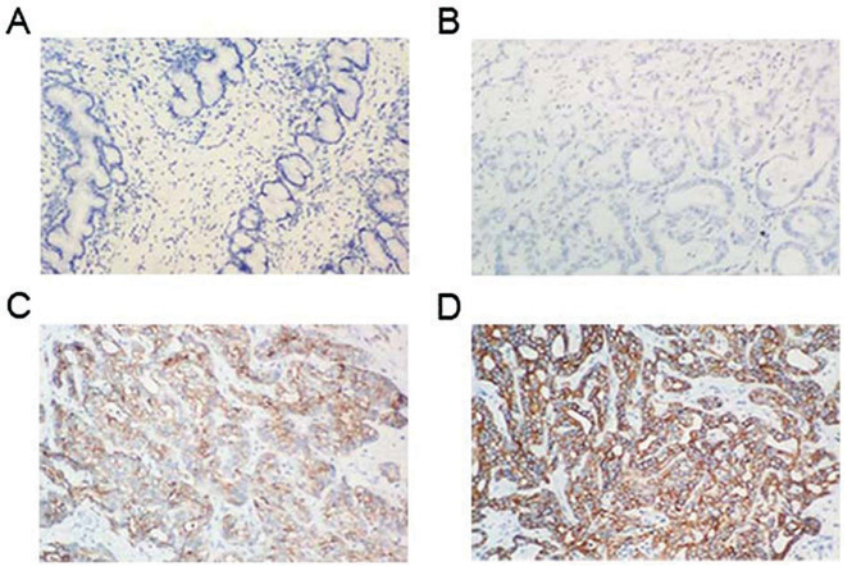

Figure 1. Immunohistochemical staining of metadherin in perihilar cholangiocarcinoma and non-neoplastic bile ducts (original magnification, x200). (A) Negative expression in normal (non-neoplastic) bile ducts. (B) Negative expression in tumor tissues. (C) Moderate expression in tumor tissues. (D) High expression in tumor tissues.

Table I. Association between MTDH protein expression in perihilar cholangiocarcinoma and clinicopathological characteristics.

		MTDH expression		
Characteristics	Number	Low (%)	High (%)	P-value
Age				0.455
≤55 years	32	18 (56.3)	14 (43.8)	
>55 years	34	16 (47.1)	18 (52.9)	
Sex				0.249
Female	21	13 (61.9)	8 (38.1)	
Male	45	21 (46.7)	24 (53.3)	
Tumor diameter				0.177
<3 cm	24	15 (62.5)	9 (37.5)	
≥3 cm	42	19 (45.2)	23 (54.8)	
Differentiation				0.007
Well/moderately	36	24 (66.7)	12 (33.3)	
Poorly	30	10 (33.3)	20 (66.7)	
Lymph node metastasis				0.023
No	28	19 (67.9	9 (32.1)	
Yes	38	15 (39.5)	23 (60.5)	
pT status				0.068
T1/T2	17	12 (70.6)	5 (29.4)	
T3/T4	49	22 (44.9)	27 (55.1)	
Nerve invasion				0.088
Negative	38	23 (60.5)	15 (39.5)	
Positive	28	11 (39.3)	17 (60.7)	
Disease stage				0.339
I/II/III	18	117 (61.1)	7 (38.9)	
IV	48	237 (47.9)	25 (52.1)	

MTDH, metadherin.

high MTDH expression was noted in the PCCA tumor tissues compared with the normal tissues. The MTDH positive expression rate was 48.5% (32/66) in PCCA tumor tissues.

Association of MTDH overexpression with clinicopathological data. The association between MTDH expression levels and PCCA clinicopathological parameters is summarized in Table I. High MTDH expression in PCCA was revealed to be positively associated with tumor differentiation and lymph node metastasis. Overexpression of MTDH occurred significantly more frequently in patients with cancer with regional lymph nodes metastasis (60.5%) compared with patients with cancer with N0-stage tumors (32.1%; P=0.023). It is important to note that MTDH expression was significantly higher in poorly differentiated PCCA (66.7%) compared with that observed in well differentiated PCCA (33.3%; P=0.007). However, no significant associations were identified with patient age, sex, tumor diameter, tumor grade and tumor classification stage.

MTDH overexpression in patients with PCCA is associated with the expression of EMT markers. The association between MTDH expression and the PCCA EMT was analyzed. An immunohistochemical method was employed in order to detect E-cadherin and vimentin expression (Fig. 2). The results indicated that high MTDH expression was significantly positively associated with vimentin expression levels in PCCA tissues compared with negative vimentin expression levels (P=0.037). However, a significant inverse association was noted between MTDH expression and positive E-cadherin compared with negative E-cadherin expression (P=0.030; Table II).

MTDH expression is associated with a poor prognosis in patients with PCCA. The 1-, 3- and 5-year OS rates were revealed to be 64.7, 44.1 and 17.6%, respectively, in the low MTDH expression group. However, in the high MTDH expression group, the 1-, 3- and 5-year OS rates were 40.6, 15.6 and 0.0%, respectively (Fig. 3A). The 1-, 3- and 5-year RFS rates

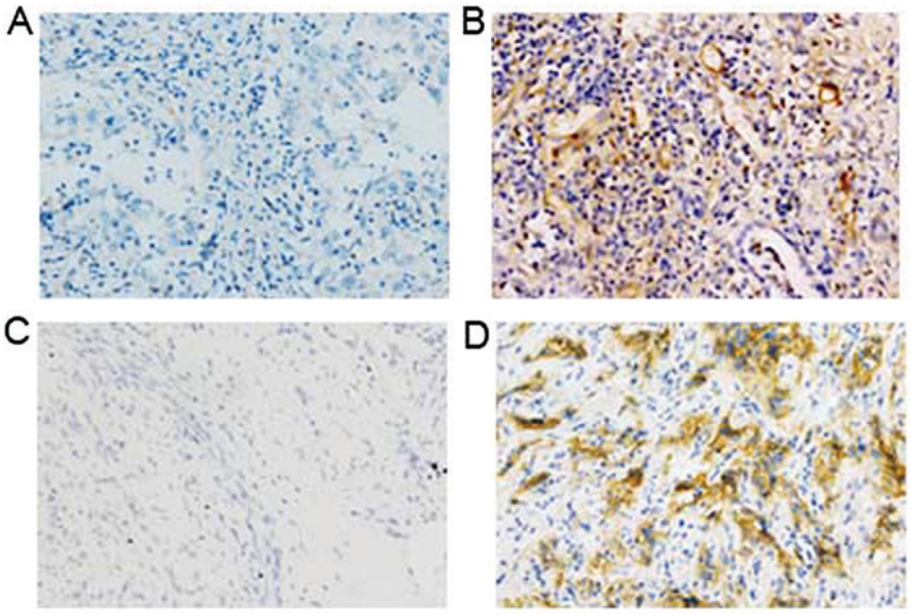

Figure 2. Immunohistochemical staining of E-cadherin and vimentin in PCCA tissues (original magnification, x200). (A) Negative expression of vimentin in PCCA tissues. (B) Positive expression of vimentin in PCCA tissues. (C) Negative expression of vimentin in PCCA tissues. (D) Positive expression of vimentin in PCCA tissues. PCCA, perihilar cholangiocarcinoma.

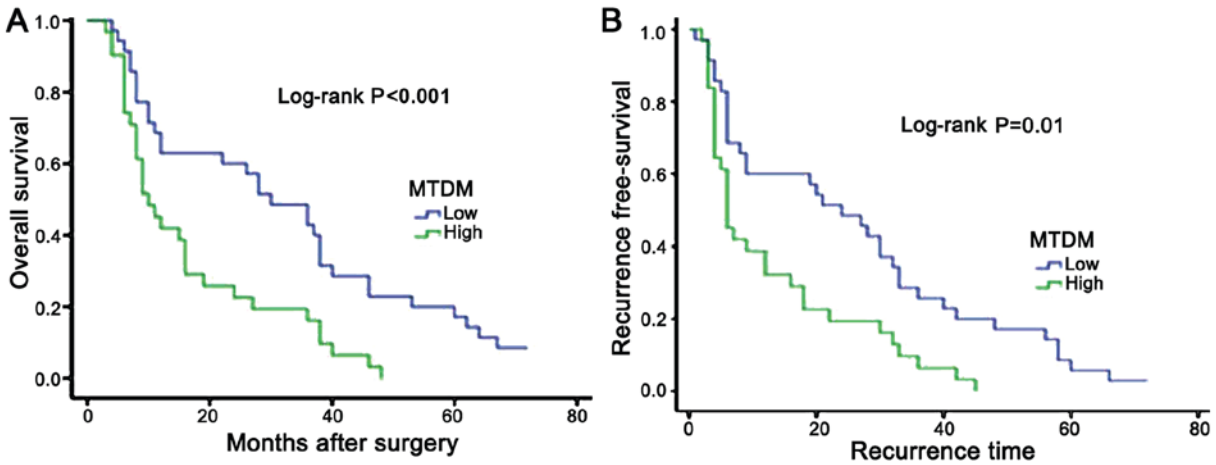

Figure 3. Kaplan-Meier analysis of OS and RFS rates in patients according to the expression of MTDH in perihilar cholangiocarcinoma tissues. Patients with high/moderate MTDH had worse (A) OS and (B) RFS rates compared with patients with low/negative MTDH expression. MTDH, metadherin; OS, overall survival; RFS, recurrence-free survival.

Table II. Association between MTDH protein expression in perihilar cholangiocarcinoma and EMT markers.

		MTDH expression		
EMT	Number	Low (%)	High (%)	P-value
E-cadherin				0.030
Positive	23	15 (65.2)	8 (34.8)	
Negative	43	16 (37.2)	27 (62.8)	
Vimentin				0.037
Positive	39	13 (33.3)	26 (66.7)	
Negative	27	16 (59.3)	11 (40.7)	

MTDH, metadherin; EMT, epithelial-to-mesenchymal transition.

were 55.9, 26.5 and 8.8%, respectively, in the low MTDH expression group. However, in the high MTDH expression group, the 1-, 3- and 5-year RFS rates were 31.3, 9.4 and 0.0%, respectively (Fig. 3B). Kaplan Meier analysis and a log-rank test suggested that the high MTDH expression group exhibited significantly worse OS and RFS rates compared with the low MTDH expression group (P<0.001 and P=0.01, respectively).

Using univariate factor analysis, it was demonstrated that tumor differentiation, tumor degree, American Joint Committee on Cancer stage (15,16), MTDH expression and surgery margin were associated with the OS and RFS. Lymph node metastasis was also associated with RFS in PCCA. In contrast to univariate analysis, Cox's analysis indicated that patients with stage IV TNM (P=0.023), high MTDH expression (P=0.030) and a surgery margin (P=0.042) were significant independent

Table III. Univariate and multivariate analysis of different parameters in perihilar cholangiocarcinoma overall survival rates by Cox's proportional hazard model.

	Univariate survival analysis			Multivariate survival analysis		
Parameters	HR	(95% CI)	P-value	HR	(95% CI)	P-value
Age (>55/≤55)	1.307	0.791-2.159	0.296			
Sex (male/female)	0.686	0.402-1.170	0.166			
Differentiation	1.820	1.061-3.123	0.030	1.580	0.906-2.757	0.107
Tumor diameter (≥3/<3)	1.212	0.720-2.040	0.118			
Tumor degree (T3-4/T1-2)	1.605	0.887-2.906	0.019	0.918	0.483-1.748	0.795
Lymph node metastasis (yes/no)	1.667	0.972-2.858	0.064			
Tumor-Node-Metastasis [IV/(I/II/III)]	2.307	1.124-3.694	0.019	2.030	1.105-3.729	0.023
Nerve invasion	1.453	0.871-2.427	0.153			
Tumor recurrence (yes/no)	1.584	1.039-2.414	0.249			
Metadherin expression (high/low)	2.438	1.433-4.147	0.001	1.736	1.065-3.388	0.030
Surgery margin (yes/no)	2.292	1.330-3.950	0.003	1.308	1.023-3.326	0.042

HR, hazard ratio; CI, confidence interval.

Table IV. Univariate and multivariate analysis of different parameters in perihilar cholangiocarcinoma recurrence-free survival rates by Cox proportional hazard model.

	Univariate survival analysis			Multivariate survival analysis		
Parameters	HR	(95% CI)	P-value	HR	(95% CI)	P-value
Age (>55/≤55)	1.290	0.784-2.122	0.316			
Sex (male/female)	0.674	0.395-1.148	0.146			
Differentiation	1.759	1.032-2.999	0.038	1.527	0.887-2.626	0.126
Tumor diameter (≥3/<3)	1.246	0.749-2.070	0.397			
Tumor degree (T3-4/T1-2)	1.499	0.847-2.652	0.164			
Lymph node metastasis (yes/no)	1.844	1.067-3.187	0.028	1.142	0.639-2.040	0.654
Tumor-Node-Metastasis [IV/(I/II/III)]	2.037	1.146-3.620	0.015	2.033	1.128-3.662	0.018
Nerve invasion	1.569	0.937-2.627	0.087			
Metadherin expression (high/low)	2.466	1.440-4.223	0.001	1.853	1.025-3.352	0.041
Surgery margin (yes/no)	2.296	1.326-3.974	0.003	1.809	1.018-3.214	0.043

HR, hazard ratio; CI, confidence interval.

prognostic factors of the OS in multivariate factors analysis (Table III). Furthermore, tumor patients with stage IV TNM (P=0.018), high MTDH expression (P=0.041) and a surgery margin (P=0.043) were also significant independent prognostic factors of RFS in patients with PCCA (Table IV).

Discussion

PCCA is a relatively rare bile duct tumor, which accounts for 2% of all cancer types identified in humans (17). The incidence of PCCA in Asian countries appears to be associated with liver infection caused by parasites from the Opistorchiidae family, while in Western countries PCCA is caused by chronic bile duct inflammation, notably primary sclerosing cholangitis (18). Surgical removal of the tumor is still considered the most effective treatment method for PCCA. Approximately 60% of patients with PCCA have a considerably wide margin resection (19). Adjuvant therapy methods including chemical drug treatment and radiotherapy may be applied in PCCA treatment and have exhibited satisfactory curative effects (20). However, the prognosis of PCCA remains very poor and even radical surgery (R0 resection) cannot increase the 5-year survival rate considerably. The 5-year survival rate of PCCA is ~40%, the recurrence rate is as high as 50-70%, and in R1/2 resection the 5-year survival rate is almost zero (21). Consequently, it is urgent to investigate the molecular mechanism underlying PCCA progression and provide novel therapies for its treatment.

In previous years, MTDH has been proposed to possess oncogenic functions by various studies (8,22-24). High MTDH

expression was associated with poor clinical pathological characteristics in the patients in the present study, including tumor stage, lymphatic metastasis, tumor recurrence and disease prognosis. High MTDH expression may result in tumorigenesis via the increased expression of phosphorylated (p-) protein kinase B, p-MDM2 proto-oncogene and p-glycogen synthase kinase-3β (GSK-3β) and via the inhibition of P53 and P21 expression (25). In liver cancer, exogenous MTDH expression in HepG3 cells indicated strong activity of mitogen-activated protein kinases, including activated extracellular signal-regulated kinase and p38. These enzymes inactivated GSK-3β through phosphorylation, and increased β-catenin phosphorylation and nuclear translocation. In this manner MTDH is able to activate the Wnt/β-catenin signaling pathway and promote tumor gene expression (26-29). The downregulation of the expression of the tumor suppressor gene phosphatase and tensin homolog, and the promotion of B-cell lymphoma 2 expression by MTDH were also reported as important anticancer mechanisms required for the treatment of human breast cancer (30). These data suggested that MTDH serves an important function in tumor development and may be considered a potential therapeutic target.

In the present study, the MTDH expression in 66 tissues of PCCA were examined using immunohistochemical methods, and it was revealed that MTDH was positive in 48.5% of PCCA tissues. Further analysis revealed that positive MTDH expression was associated with lymph node metastasis and poor differentiation in patients with PCCA. Survival prognostic analysis suggested that a high MTDH expression in PCCA resulted in a worse RFS and OS rates. Although the number of clinical samples was limited, the included patients were only 66 cases in the present study, and the results suggested that high MTDH expression in PCCA may provide a meaningful tumor marker that is able to predict patient prognosis.

Previous studies have clearly demonstrated that MTDH participates in breast cancer metastasis to the lung (8,31,32). In addition, MTDH was reported to have an effect in promoting tumor metastasis in a number of human cancer types (33,34). EMT is an important characteristic for the initiation of tumor cell migration and invasion. One previous study demonstrated that MTDH participates in EMT by upregulating N-cadherin, Snail family transcriptional repressor 1 and Snail family transcriptional repressor 2 expression and by inhibiting E-cadherin expression (35). It has been further reported that certain microRNAs (miRNAs) may regulate EMT by targeting MTDH (18). These results are consistent with the analysis in the present study and indicate that high MTDH expression in PCCA is highly associated with lymph node metastasis.

The oncogenic function of MTDH may be associated with with tumor metastasis for various reasons. Firstly, MTDH may promote angiogenesis through enhancing the expression of multiple angiogenesis molecular markers (36). Secondly, MTDH may inhibit the expression of cell cycle protein inhibitors, including p53, p21 and p27 and induce the expression of cell cycle promoting proteins, including cyclin D1 and cyclin E (37). In prostate cancer, the inhibition of MTDH promoted apoptosis, reduced cell viability and increased cell sensitivity to cisplatin (38). MTDH may also promote human cancer growth by regulating the expression of specific miRNAs (39). In contrast to the present study, miRNA-630 may inhibit breast cancer cell growth by targeting MTDH (40). In the present study, it was demonstrated that high MTDH expression was associated with poor differentiation in PCCA. However, no significant difference was noted with regard to high MTDH expression and patients with PCCA with a large tumor diameter compared with small tumor diameter. These results strongly suggest that MTDH may promote PCCA growth and malignant transformation through multiple mechanisms of action. Further experiments including analyzing the association of MTDH and Ki67 in tumor tissues and using *in vivo* experiments to confirm the importance of MTDH expression are required.

The present study demonstrated that high MTDH expression was noted in PCCA cases, and that high MTDH expression in patients with PCCA was associated with poor tumor differentiation, lymph node metastasis and a worse disease prognosis. MTDH may serve a vital function in promoting malignant transformation and is a potential therapeutic target in the treatment of PCCA. To the best of our knowledge, this is the first study to report MTDH expression in PCCA and its association with the clinicopathological characteristics of this disease. The detailed molecular mechanisms of the function of MTDH in PCCA require further investigation.

In the present study, MTDH expression in patients with PCCA was investigated and attempted to determine its clinical and pathological significance. It was demonstrated that high MTDH expression may serve an important function in PCCA tumor growth and metastasis, and thus targeting MTDH potentially has important therapeutic applications for patients with PCCA.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

YZ and WFL performed the experiments and wrote the manuscript. WFL made substantial contributions to conception and design of the manuscript. YZ was responsible for the design of the experiments. KZ and FS analyzed the experimental data. LLR and WFL assisted with the statistical analysis. GW critically revised the manuscript and provided final approval of the version to be published and also made substantial contributions to conception and design. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of the Taihe Hospital Affiliated to Hubei University of Medicine (Hubei, China) and written informed consent was obtained from each patient prior to enrolment.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that there are no competing interests.

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