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Combined OPCML and AXL Expression as a Prognostic Marker and OPCML Enhances AXL Inhibitor in Cholangiocarcinoma

RICUPHAN KHAMKO[1,2], WIPHAWAN WASENANG[3], JUREERUT DADUANG[2], CHATRI SETTASATIAN[4] and TEMDUANG LIMPAIBOON[2,5]

[1]*Biomedical Science Program, Graduate School, Khon Kaen University, Khon Kaen, Thailand;*
[2]*Centre for Research and Development of Medical Diagnostic Laboratories, Faculty of Associated Medical Sciences, Khon Kaen University, Khon Kaen, Thailand;*
[3]*Faculty of Medical Technology, Nakhon Ratchasima College, Nakhon Ratchasima, Thailand;*
[4]*Department of Pathology, Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand;*
[5]*Cholangiocarcinoma Research Institute, Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand*

Abstract. *Background/Aim: Cholangiocarcinoma (CCA) is a type of liver cancer originating from bile duct epithelium which has an unfavorable prognosis. Therefore, novel prognostic markers and effective therapeutic regimens are required. Opioid-binding protein/cell adhesion molecule-like (OPCML) is a tumor-suppressor protein that suppresses CCA cell proliferation via AXL receptor tyrosine kinase/signal transducer and activator of transcription 3 (AXL/STAT3) inactivation. However, this association in clinical samples remains unknown. We aimed to determine OPCML and AXL expression and investigate their association with clinicopathological features in patients with CCA. In addition, we also addressed whether OPCML enhanced the sensitivity of CCA cells to AXL inhibitor R428 in vitro. Materials and Methods: The expression of OPCML and AXL was determined by immunohistochemistry in 90 CCA tissue samples. The study of CCA cell line sensitivity to R428 was performed by cell viability assay. Results: The expression of OPCML was significantly lower while AXL expression was substantially higher in CCA than in adjacent normal tissue (p<0.001). Furthermore, high AXL expression was significantly associated with lymph node metastasis (p=0.035). Interestingly, patients with combined low OPCML/high AXL expression had significantly shorter overall survival (p=0.007). OPCML enhanced the effect of AXL inhibitor R428 in AXL-expressing CCA cell lines. Conclusion: Combined expression of OPCML and AXL shows potential value as a prognostic marker and OPCML as an agent enhancing the effect of R428 may contribute to better prognosis for patients with CCA.*

Correspondence to: Temduang Limpaiboon, Centre for Research and Development of Medical Diagnostic Laboratories, Faculty of Associated Medical Sciences, Khon Kaen University, Khon Kaen 40002, Thailand. E-mail: temduang@kku.ac.th

Key Words: OPCML, AXL, prognostic marker, AXL inhibitor R428, enhancing agent.

Cholangiocarcinoma (CCA) is a malignancy that arises from bile duct epithelial cells (1). CCA is rare worldwide but common in Southeast Asia, particularly in Northeastern Thailand, and is related to liver fluke (*Opisthorchis viverrini*) infection, which is known to be a strong risk factor for CCA in this region (2). Nowadays, CCA accounts for about 2% of cancer-related deaths per year worldwide. Moreover, it is characterized by a poor prognosis due to a lack of early detection and its low response rate to existing chemotherapy (3). Accordingly, it is crucial to find out novel biomarkers and therapeutic strategies to improve CCA prognosis.

Opioid-binding protein/cell adhesion molecule-like (OPCML) is a glycosyl phosphatidylinositol-anchored cell adhesion-like molecule which belongs to a member of the IgLON subfamily in the immunoglobulin superfamily of cell adhesion molecules of proteins (4). Normally, OPCML plays roles in a cell adhesion and is a negative regulator. Moreover, OPCML also acts as a tumor suppressor which is often inactivated by DNA hypermethylation, resulting in gene silencing in several cancer types (5-8), including CCA (9).

Previous studies revealed the crucial functions of OPCML in the inhibition of progression of many types of human

cancer (4, 8, 10-13). Recently, Antony *et al*. reported that AXL receptor tyrosine kinase (AXL) was a potential target molecule of OPCML, to which OPCML directly binds, resulting in the repression of ovarian cancer cell migration and invasion (12). AXL is a member of the TYRO3 protein tyrosine kinase, AXL and MER proto-oncogene tyrosine kinase receptor tyrosine kinase family (14). Activation of AXL has been related to tumor growth and metastasis of various cancer types (15). Furthermore, our recent study demonstrated that ectopic expression of OPCML suppressed CCA cell proliferation through the inactivation of AXL/signal transducer and activator of transcription 3 (STAT3) signaling pathway (13), indicating the association between OPCML and AXL in CCA progression. However, this association has not been investigated in clinical samples.

Herein, we aimed to study the expression of OPCML and AXL by immunohistochemistry in CCA tissue samples. The association of OPCML and AXL expression with clinicopathological parameters was evaluated to address their potential as novel prognostic markers in CCA. As an approach to finding new therapeutic regimens for CCA, we also determined whether ectopic expression of OPCML influenced the effect of AXL inhibitor R428 in AXL-expressing CCA cell lines.

Materials and Methods

Patients and samples. This study was approved by the Khon Kaen University Ethics Committee for Human Research (HE641063). Written informed consent for use of their tissues and data was received from all patients. Paraffin-embedded tissue sections of 90 CCA and 86 matched adjacent normal samples were recruited in this study. The clinicopathological data including sex, age, lymph node metastasis, tumor grading, tumor gross type, tumor size, tumor location and survival time (no survival data were available from three patients and the survival data from three patients who died within 4 weeks after surgery were excluded) were kindly provided by the Cholangiocarcinoma Research Institute, Khon Kaen University, Khon Kaen, Thailand.

Immunohistochemical (IHC) staining and scoring. Paraffin-embedded tissue sections at 4-μm thickness were deparaffinized and rehydrated. Antigen retrieval was carried out by boiling the sections in 0.01 M citrate buffer (pH 6.0) for 10 min. Endogenous peroxidase enzyme was blocked by 3% H_2O_2 for 1 h in the dark. To block non-specific binding, the sections were treated with 20% fetal bovine serum for 2 h. The sections were then incubated overnight at 4˚C in a humidified chamber with 1:1,000 rabbit monoclonal anti-OPCML (Abcam, Cambridge, UK) or 1:150 rabbit monoclonal anti-AXL (C89E7) (Cell Signaling Technology, Danvers, MA, USA); a negative control (antibody diluent instead of primary antibody) was also performed. After incubation, the sections were washed twice with phosphate-buffered saline containing 0.05% Tween-20 for 10 min for each washing before being incubated with goat anti-rabbit secondary antibody (Envision™ System; DAKO Corporation, Carpinteria, CA, USA) for 1 h at room temperature. Antigen–antibody reaction was visualized using diaminobenzidine chromogen (DAKO Corporation) and the sections were then counterstained with Mayer's hematoxylin. Protein expression was examined and scored by two independent observers using a semi-quantitative IHC scoring system (16). H-Scores were defined as the sum of the stained cell percentage multiplied by an ordinal value corresponding to the intensity level (0=none, 1=weak, 2=moderate, 3=strong), and ranged from 0 to 300. To define H-scores as representing low or high expression for each protein, the mean H-score for OPCML and median H-score for AXL were used as optimal cut-offs for clinicopathological data analysis.

Table I. *List of primers used in this study.*

Primer		Sequence (5'→3')	Product size (bp)
AXL	Forward	ACGTATCGCCAAGATGCCAG	252
	Reverse	GGATTTAGCTCCCAGCACCG	
GAPDH	Forward	AGAGGCAGGGATGATGTTCT	243
	Reverse	ATGTTCGTCATGGGTGTGAA	

AXL: AXL receptor tyrosine kinase; *GAPDH*: glyceraldehyde 3-phosphate dehydrogenase; bp: base pair.

Cell lines and culture. CCA (KKU-M213A, KKU-M055 and KKU-100) and immortalized cholangiocyte (MMNK-1) cell lines were kindly provided by the Cholangiocarcinoma Research Institute, Khon Kaen University, Thailand. KKU-M055, KKU-M213A and MMNK-1 were grown in Dulbecco's modified Eagle's medium, while KKU-100 was grown in Ham's F-12 Nutrient Mixture medium, which included 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin (all from Thermo Fisher Scientific, Waltham, MA, USA). Cell culture was maintained at 37˚C with 5% CO_2.

AXL inhibitor study. After transient transfection for 48 h with *OPCML* and control plasmids as described in our previous study (13), transfected KKU-M213A and KKU-100 (5×10^3 cells) in 100 μl of complete medium were seeded in a 96-well plate in triplicate. After 18 h incubation, the cells were then treated with different concentrations (0, 2, 4, 8, 16 and 32 μM) of AXL inhibitor R428 (Abcam) for 48 h. Cell viability was then assessed using CellTiter 96® Aqueous One Solution Cell Proliferation Assay (Promega) according to the manufacturer's instructions, from which the 50% inhibitory concentration (IC_{50}) was generated.

Quantitative reverse transcription–polymerase chain reaction (qRT-PCR). To screen endogenous expression of *AXL* mRNA, TRIzol reagent (Thermo Fisher Scientific) was used to extract total RNA from the native cell lines, which was subsequently synthesized to cDNA using the ImProm-II™ Reverse Transcription System kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. Table I shows PCR primers for human *AXL* and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) as an internal control. Real-time PCR was carried out on a Rotor-Gene Q analyzer (QIAGEN, Hilden, Germany) as follows: An initial denaturation at 93˚C for 5 min, followed by 35 cycles of the following steps: 93˚C for 30 s, 54˚C for *AXL* or 58˚C for *GAPDH* for 30 s, and 72˚C for 30

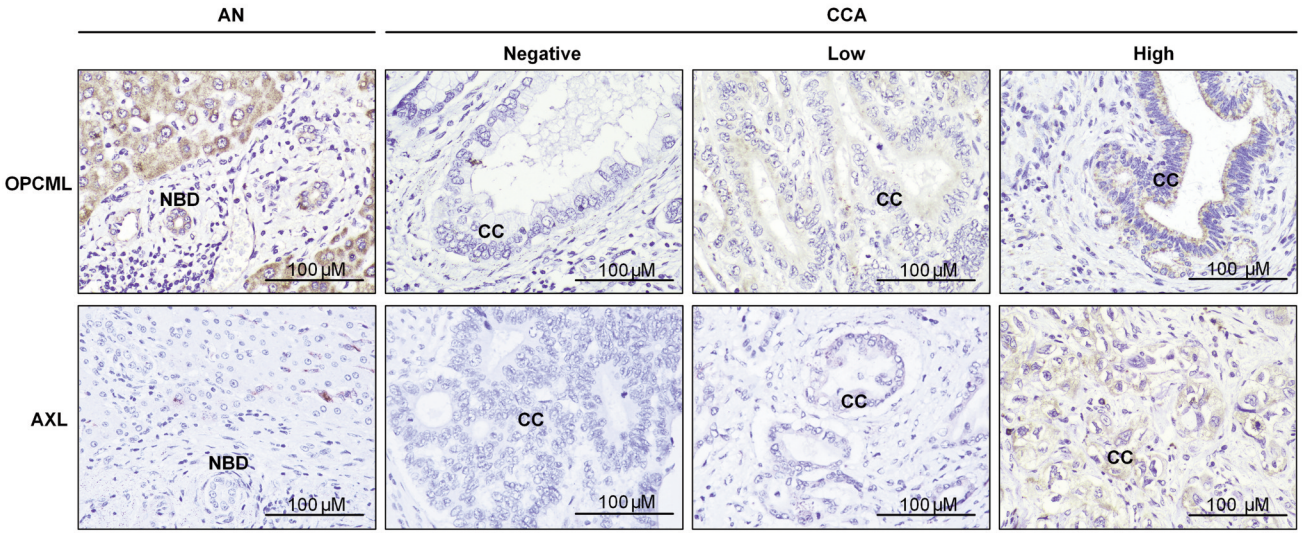

Figure 1. *Representative immunohistochemical staining of opioid-binding protein/cell adhesion molecule-like (OPCML) and AXL receptor tyrosine kinase (AXL) in tissues from patients with cholangiocarcinoma (CCA). Representative staining of CCA and adjacent normal tissue (AN) are shown. Original magnification, ×400. CC: Cancerous cells; NBD: normal bile duct.*

s, with a final elongation at 72˚C for 10 min. The PCR product was quantified by the incorporation of SYTO 9 dye (Thermo Fisher Scientific). Relative gene expression was analyzed by the comparative Ct method ($2^{-\Delta\Delta Ct}$), where $\Delta Ct=(Ct_{target}-Ct_{reference})$ (17).

Western blot analysis. Cell lysates from representative native and *OPCML*-transfected CCA cell lines were prepared using radioimmunoprecipitation assay buffer (Cell Signaling Technology) containing protease and phosphatase inhibitors (Roche, Basel, Switzerland) in order to investigate endogenous AXL expression and its expression after being transfected by *OPCML* plasmid, respectively. Protein concentrations were determined by Coomassie Plus-The Better Bradford Assay™ kit (Thermo Fisher Scientific). The protein lysates (20 μg) were separated on 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes. The membranes were blocked with 5% skim milk in Tris-buffered saline with 0.1% Tween-20 for 1 h and subsequently probed with primary antibodies overnight at 4˚C. The antibodies used were as follows: 1:1,000 rabbit monoclonal anti-AXL (C89E7) (Cell Signaling Technology), 1:1,000 rabbit monoclonal anti-OPCML (Abcam) or 1:10,000 rabbit polyclonal anti-actin (Abcam). The membranes were washed three times, then incubated with 1:2,000 donkey anti-rabbit IgG (Abcam) for 1 h. After washing three times, ECL™ reagents (GE Healthcare, Chicago, IL, USA) were used to detect proteins. The protein images were qualitatively analyzed by Amersham Imager 600 (GE Healthcare).

Statistical analysis. The comparison of H-scores between two groups was analyzed using independent-sample *t*-test for OPCML and Mann-Whitney test for AXL expression. Values are presented as the mean±standard deviation or median (min-max). The correlation between clinicopathological data and protein expression in CCA was evaluated using Pearson chi-squared test. Overall survival curves were produced using Kaplan-Meier method and log-rank test. The Cox regression model was included for univariate and multivariate analyses. A value of $p<0.05$ was considered as statistically significant. All statistical analyses were performed using SPSS version 25 software (IBM, Armonk, NY, USA).

Results

Down-regulation of OPCML and up-regulation of AXL in CCA. The expression of OPCML and AXL was assessed by IHC in 90 CCA and 86 matched adjacent normal tissues and presented as H-scores. Representative IHC staining of OPCML and AXL in the tissue samples is shown in Figure 1. OPCML expression in CCA was significantly lower than that in adjacent normal tissue ($p<0.001$), whereas that of AXL was up-regulated in CCA ($p<0.001$) (Figure 2A). As shown in Figure 2B, high AXL expression was predominantly observed in metastatic CCA ($p=0.015$), but OPCML expression did not differ according to metastasis ($p=0.448$).

Correlation of clinicopathological parameters with expression of OPCML and AXL in patients with CCA. The H-scores for OPCML and AXL were evaluated for cut-off values using the mean and median, respectively, and were used to classify patients with CCA into two groups with low (n=45) and high (n=45) expression for each protein. The correlation between the expression levels of OPCML and AXL, and clinicopathological parameters including sex, age, lymph node metastasis, tumor grading, tumor gross type, tumor size and tumor location was then analyzed. As shown in Table II, OPCML expression was not significantly correlated with any parameter. In contrast, high expression of AXL was significantly related to lymph node metastasis ($p=0.035$).

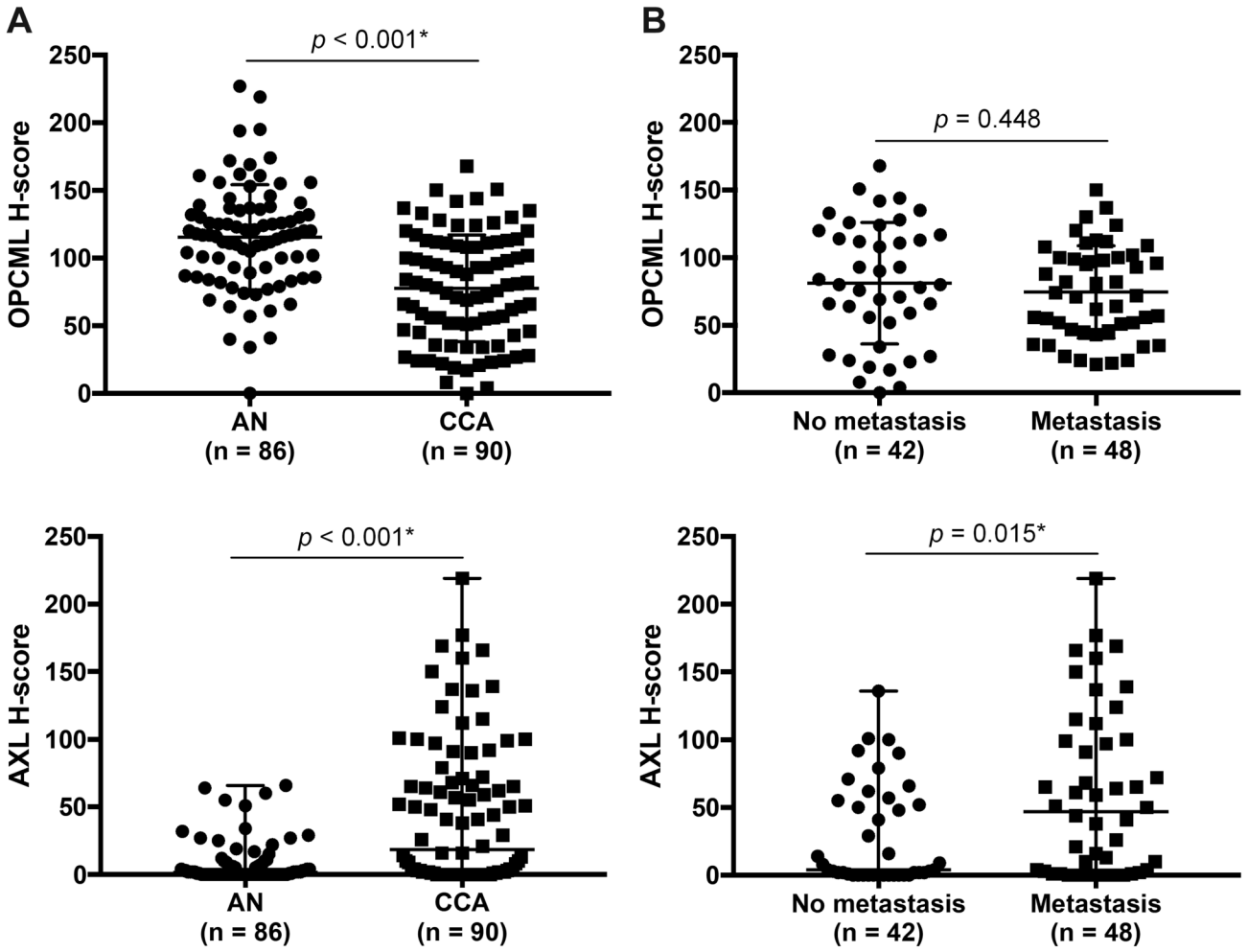

Figure 2. *Scatter plots of immunohistochemical scores (H-scores) for opioid-binding protein/cell adhesion molecule-like (OPCML) and AXL receptor tyrosine kinase (AXL) in adjacent normal tissue (AN) (n=86) and cholangiocarcinoma (CCA) (n=90) (A), and non-metastatic CCA (n=42) and metastatic CCA (n=48) (B). The difference between two groups was analyzed by independent-sample t-test for OPCML and Mann-Whitney test for AXL. *Statistically significantly different.*

Correlation between clinicopathological features, and combined detection of OPCML and AXL expression levels in CCA patients. Ninety patients with CCA were classified into four groups: Low OPCML/low AXL (n=23), low OPCML/high AXL (n=22), high OPCML/low AXL (n=22) and high OPCML/high AXL (n=23). The relationship between the combined OPCML and AXL expression levels, and clinical features was examined in Table III. Significant association for combined expression of OPCML/AXL and tumor gross type was observed (p=0.028). Unfortunately, association with specific tumor gross type could not be defined by chi-squared test alone.

High AXL and combined low OPCML/high AXL expression were associated with shorter overall survival in patients with CCA. The relationship of the expression levels of OPCML, AXL alone and combined with overall survival was evaluated by Kaplan-Meier analysis. No difference in overall survival was observed between patients with low and those with high OPCML expression (p=0.138) (Figure 3A). By contrast, patients with high AXL expression had significantly shorter overall survival than those with low expression (p=0.004) (Figure 3B). Interestingly, patients with combined low OPCML/high AXL expression had significantly lower overall survival than those in the other groups (p=0.007) (Figure 3C). In addition, the univariate Cox regression analysis showed the association of high AXL expression with poor prognosis of CCA, especially in combination with low OPCML expression, with a hazard ratio of 2.366 (95% confidence interval=1.327-4.219; p=0.004). Hence, our

Table II. *Correlation of opioid-binding protein/cell adhesion molecule-like (OPCML) and AXL receptor tyrosine kinase (AXL) expression levels with clinicopathological parameters in patients with cholangiocarcinoma.*

		OPCML expression, n (%)			AXL expression, n (%)		
Clinical parameter	n	Low	High	*p*-Value	Low	High	*p*-Value
Sex				0.172			0.172
Male	62	34 (75.6)	28 (62.2)		34 (75.6)	28 (62.2)	
Female	28	11 (24.4)	17 (37.8)		11 (24.4)	17 (37.8)	
Age				0.396			0.671
<62 Years	40	22 (48.9)	18 (40.0)		19 (42.2)	21 (46.7)	
≥62 Years	50	23 (51.1)	27 (60.0)		26 (57.8)	24 (53.3)	
Lymph node metastasis				0.673			**0.035**
No	42	20 (44.4)	22 (48.9)		26 (57.8)	16 (35.6)	
Yes	48	25 (55.6)	23 (51.1)		19 (42.2)	29 (64.4)	
Tumor grading				0.290			0.525
Papillary	49	27 (60.0)	22 (48.9)		26 (57.8)	23 (51.1)	
Non-papillary	41	18 (40.0)	23 (51.1)		19 (42.2)	22 (48.9)	
Tumor gross type				0.551			0.212
Mass-forming	27	12 (26.7)	15 (33.3)		10 (22.2)	17 (37.8)	
Periductal infiltration	15	10 (22.2)	5 (11.1)		6 (13.3)	9 (20.0)	
Intraductal	10	5 (11.1)	5 (11.1)		6 (13.3)	4 (8.9)	
Mixed	38	18 (40.0)	20 (44.5)		23 (51.2)	15 (33.3)	
Tumor size				0.829			0.517
<4 cm	35	18 (40.0)	17 (37.8)		16 (35.6)	19 (42.2)	
≥4 cm	55	27 (60.0)	28 (62.2)		29 (64.4)	26 (57.8)	
Tumor location				0.172			0.362
Intrahepatic	62	28 (62.2)	34 (75.6)		33 (73.3)	29 (64.4)	
Extrahepatic	28	17 (37.8)	11 (24.4)		12 (26.7)	16 (35.6)	

Statistically significant *p*-values by chi-squared test are shown in bold.

findings suggest that combined low OPCML/high AXL expression might be a potential marker for predicting an unfavorable prognosis for CCA. However, after adjusting the multivariate Cox regression analysis for lymph node metastasis, only lymph node metastasis remained an independent prognostic marker for CCA with hazard ratio of 3.203 (95% confidence interval=1.720-5.964; $p<0.001$) as shown in Table IV.

OPCML enhances sensitivity of AXL-expressing CCA cells to AXL inhibitor R428. To find a novel therapeutic regimen for improving prognosis of patients with CCA, we initially determined endogenous *AXL* mRNA expression in native CCA and MMNK-1 cell lines using qRT-PCR. The results showed that *AXL* mRNA was endogenously expressed in all three native CCA cell lines when compared to immortalized MMNK-1 cells ($p<0.01$) (Figure 4A). Additionally, endogenous AXL protein expression was also observed in two representative CCA cell lines, KKU-M213A and KKU-100, by western blot analysis (Figure 4B). Because OPCML was not expressed in those two representative cell lines, OPCML was ectopically expressed in them by transient transfection as described in our previous study (13). Ectopic OPCML expression did not affect endogenous AXL expression (Figure 4C). Control-transfected cells and *OPCML*-transfected cells were treated with the AXL inhibitor R428. We found that *OPCML* transfection significantly reduced the mean IC_{50} of R428 from 9.59 to 5.67 μM (1.7-fold) in KKU-M213A ($p=0.001$) and 10.37 to 8.30 μM (1.2-fold) in KKU-100 ($p=0.008$) cells compared to their respective controls (Figure 4D). Our findings suggest that *OPCML* enhanced the effect of R428 in AXL-expressing CCA cells.

Discussion

To date, the needs in management of patients with CCA remain unmet because of difficulties in early detection (18), poor prognosis and chemotherapeutic drug resistance (19). Therefore, novel biomarkers and therapy regimens are urgently required.

OPCML was firstly discovered as a tumor-suppressor gene in epithelial ovarian cancer, in which it is often epigenetically inactivated (5). Later studies demonstrated low expression of OPCML to be related to the carcinogenesis of various cancer types including invasive cervical carcinoma (6), bladder carcinoma (7), gastric cancer (11), brain tumors (20), ovarian cancer (21), prostate cancer (22) and CCA (23). In addition,

Table III. *Correlation of the combined expression of opioid-binding protein/cell adhesion molecule-like (OPCML) and AXL receptor tyrosine kinase (AXL) with clinicopathological features in patients with cholangiocarcinoma.*

		Low OPCML, n (%)		High OPCML, n (%)		
Clinical parameter	n	Low AXL	High AXL	Low AXL	High AXL	*p*-Value
Sex						0.091
Male	62	17 (73.9)	17 (77.3)	17 (77.3)	11 (47.8)	
Female	28	6 (26.1)	5 (22.7)	5 (22.7)	12 (52.2)	
Age						0.553
<62 Years	40	12 (52.2)	10 (45.5)	7 (31.8)	11 (47.8)	
≥62 Years	50	11 (47.8)	12 (54.5)	15 (68.2)	12 (52.2)	
Lymph node metastasis						0.120
No	42	14 (60.9)	6 (27.3)	12 (54.5)	10 (43.5)	
Yes	48	9 (39.1)	16 (72.7)	10 (45.5)	13 (56.5)	
Tumor grading						0.382
Papillary	49	16 (69.6)	11 (50.0)	10 (45.5)	12 (52.2)	
Non-papillary	41	7 (30.4)	11 (50.0)	12 (54.5)	11 (47.8)	
Tumor gross type						**0.028**
Mass-forming	27	5 (21.7)	7 (31.8)	5 (22.7)	10 (43.5)	
Periductal infiltration	15	2 (8.7)	8 (36.4)	4 (18.2)	1 (4.3)	
Intraductal	10	5 (21.7)	0 (0.0)	1 (4.5)	4 (17.4)	
Mixed	38	11 (47.9)	7 (31.8)	12 (54.6)	8 (34.8)	
Tumor size						0.565
<4 cm	35	7 (30.4)	11 (50.0)	9 (40.9)	8 (34.8)	
≥4 cm	55	16 (69.6)	11 (50.0)	13 (59.1)	15 (65.2)	
Tumor location						0.170
Intrahepatic	62	17 (73.9)	11 (50.0)	16 (72.7)	18 (78.3)	
Extrahepatic	28	6 (26.1)	11 (50.0)	6 (27.3)	5 (21.7)	

Statistically significant *p*-values by chi-squared test are shown in bold.

Table IV. *Correlation of the combined expression of opioid-binding protein/cell adhesion molecule-like (OPCML) and AXL receptor tyrosine kinase (AXL) with clinicopathological features in patients with cholangiocarcinoma.*

		Univariate analysis		Multivariate analysis[a]	
Parameter	Comparison	HR (95% CI)	*p*-Value	HR (95% CI)	*p*-Value
Sex	Female *vs*. male (ref)	1.499 (0.859-2.615)	0.154	–	–
Age	≥62 *vs*. <62 Years (ref)	0.755 (0.439-1.299)	0.311	–	–
Lymph node metastasis	Yes *vs*. no (ref)	3.791 (2.084-6.894)	**<0.001**	3.203 (1.720-5.964)	**<0.001**
Tumor grading	Non-papillary *vs*. papillary (ref)	0.826 (0.479-1.422)	0.490	–	–
Tumor gross type	PI, ID, mixed *vs*. MF (ref)	1.650 (0.935-2.911)	0.084	–	–
Tumor size	<4 *vs*. ≥4 cm (ref)	1.236 (0.704-2.170)	0.461	–	–
Tumor location	Extrahepatic *vs*. intrahepatic (ref)	1.107 (0.626-1.957)	0.727	–	–
OPCML expression	High *vs*. low (ref)	0.666 (0.387-1.145)	0.141	–	–
AXL expression	High *vs*. low (ref)	2.293 (1.293-4.067)	**0.005**	1.423 (0.720-2.813)	0.310
OPCML/AXL	Low/high *vs*. low/low, high/low, high/high (ref)	2.366 (1.327-4.219)	**0.004**	1.490 (0.761-2.920)	0.245

CI: Confidence interval; HR: hazard ratio; ID: intraductal; MF: mass-forming; PI: periductal infiltration. [a]Adjusted for lymph node metastasis. Statistically significant *p*-values are shown in bold.

the roles of OPCML as a tumor suppressor in many human cancer types were also reported.

AXL is a target molecule of OPCML, which can directly interact with AXL, subsequently suppressing cancer cell motility and invasion through inactivation of AXL oncogenic signaling pathway in ovarian cancer (12). Our recent study in CCA cell lines illustrated that OPCML reduced cell proliferation by inactivation of the AXL/STAT3 signaling

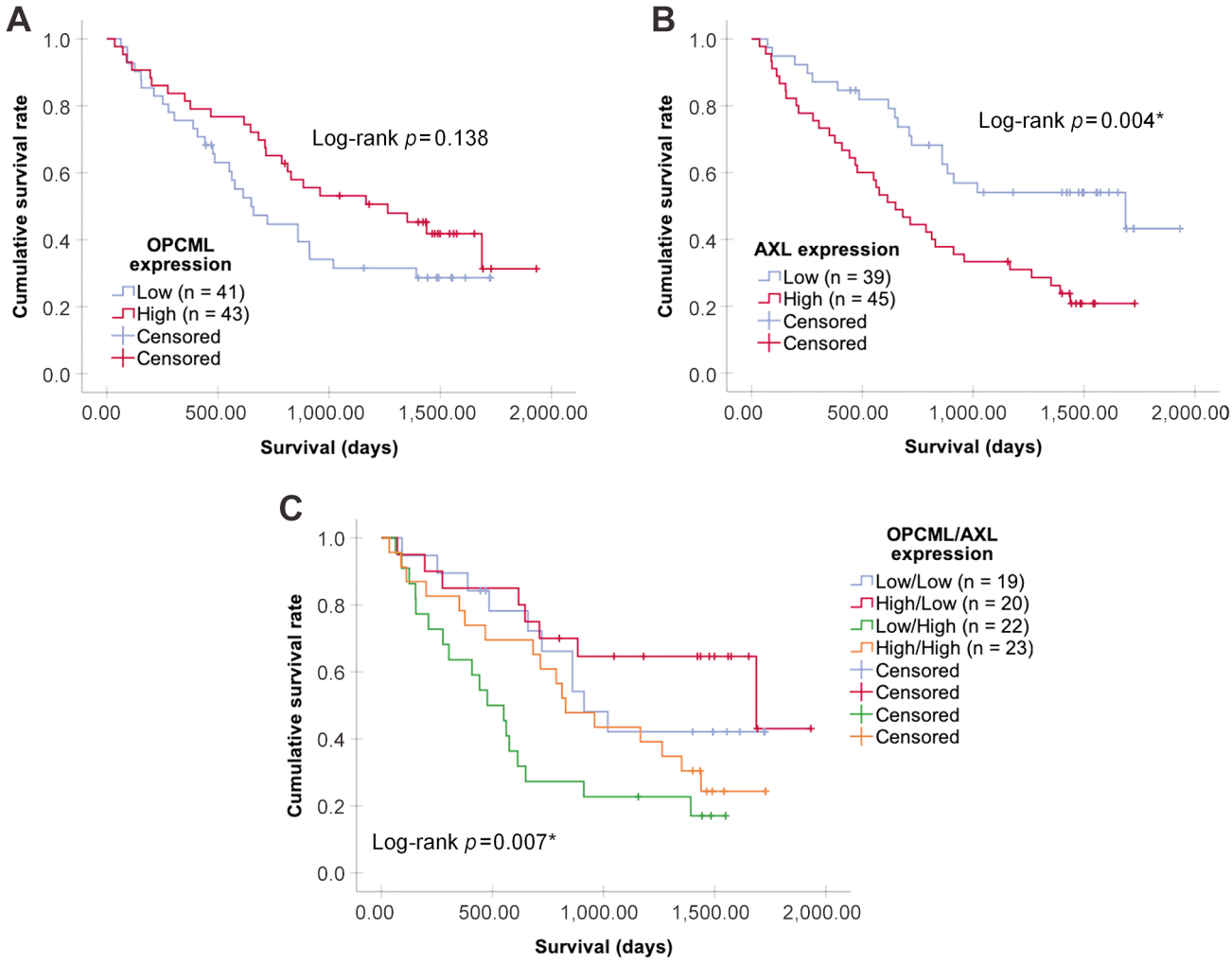

Figure 3. *Kaplan-Meier curves of survival of patients with cholangiocarcinoma according to the expression of opioid-binding protein/cell adhesion molecule-like (OPCML) (A), AXL receptor tyrosine kinase (AXL) (B) and the combination of OPCML and AXL (C). *Statistically significant by the log-rank test.*

pathway, indicating a tumor-suppressive role of OPCML and the association between OPCML and AXL during CCA progression (13). In the present study, we further investigated their association with clinical parameters in patients with CCA. We found significantly low expression of OPCML in CCA compared to adjacent normal tissue, agreeing with our recent study in CCA cell lines (13). Sriraksa *et al*. demonstrated that low or no expression of OPCML was observed mostly in *OPCML*-hypermethylated CCA tissue (9). Additionally, reduced OPCML expression was also reported by Xing *et al*. in gastric cancer tissue (11) and in breast cancer by Lian *et al*. (10). We further illustrated significantly high expression of AXL in CCA compared to adjacent normal tissue, especially in metastatic CCA. Metastasis is a key driver of tumor progression. Alvarez *et al*. suggested that AXL protein was highly expressed in approximately 60% of primary esophageal adenocarcinoma and in a comparable percentage of lymph node metastases (24). In addition, Zhao *et al*. also reported significantly high expression of AXL in non-small-cell lung cancer compared to paracancerous lung tissue (25). Moreover, high AXL expression was related to brain metastasis (26).

Low expression of OPCML observed in our study was not associated with short overall survival. In contrast, high expression of AXL was correlated with short overall survival in patients with CCA, in agreement with other studies in many cancer types (24, 25, 27). Lymph node metastasis as an independent prognostic marker for CCA was noted in this study, which was in concordance with a previous study (28). Interestingly, the combination of low

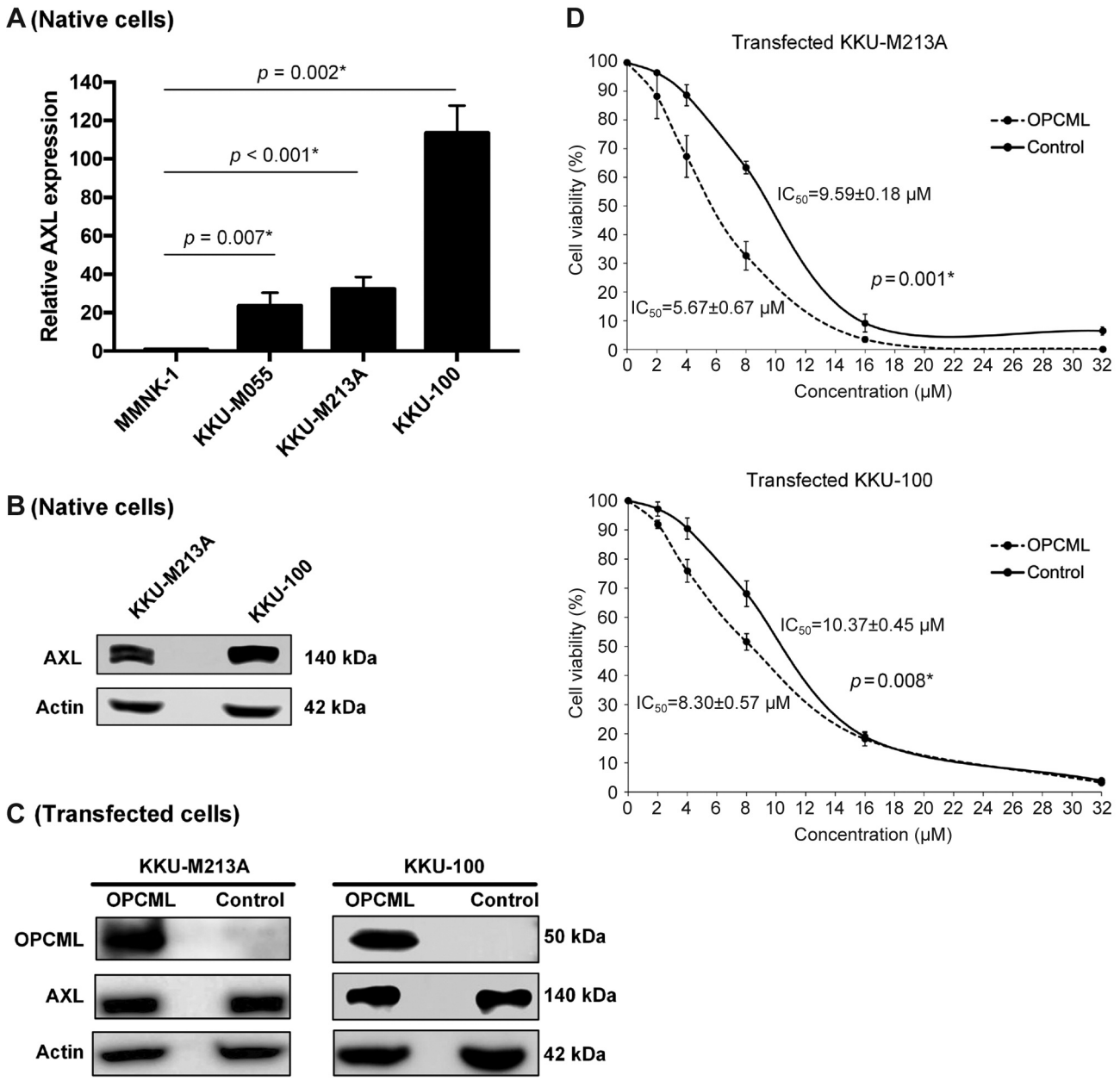

Figure 4. *Ectopic opioid-binding protein/cell adhesion molecule-like (OPCML) enhanced the effect of AXL receptor tyrosine kinase (AXL) inhibitor R428 in representative AXL-expressing cholangiocarcinoma cell lines. A: Determination of endogenous AXL mRNA expression by quantitative reverse transcription–polymerase chain reaction. Values are presented as the mean±standard deviation from independent experiments running in triplicate. B: Endogenous AXL protein expression was detected by western blot analysis. C: Ectopic expression of OPCML had no effect on endogenous AXL expression in OPCML-transfected cells. D: Effect of OPCML transfection on cytotoxicity of AXL inhibitor R428 in cell viability assays. The 50% inhibitory concentration (IC_{50}) of R428 in control and OPCML-transfected cells is presented as the mean±standard deviation from three independent experiments running in triplicate. Ectopic expression of OPCML significantly reduced the IC_{50}. *Statistically significant.*

OPCML and high AXL expression was significantly associated with an unfavorable prognosis, suggesting the contribution of OPCML and AXL in CCA progression. The data from patients with CCA in the present study and cell lines in our previous study (13) indicate the involvement of OPCML in the regulation of AXL. As expected, OPCML enhanced the sensitivity of AXL-expressing CCA cells to AXL inhibitor R428. Our study agreed with another study in ovarian cancer (12), suggesting this to be a promising approach for CCA treatment.

R428 (BGB324) is a highly potent and frequently studied AXL inhibitor which blocks AXL autophosphorylation at its *C*-terminal docking site, Tyr821. R428 inhibits cancer cell growth and metastasis, and induces apoptosis (29). Moreover, the relationships between the crystal structure and functions of OPCML were recently demonstrated, giving rise to the notion of developing OPCML as a potent anticancer drug (30). Thus, the administration of OPCML and AXL inhibitor R428 in patients with CCA with low OPCML/high AXL expression might be a novel treatment regimen for prognostic improvement.

To our knowledge, this is the first report to describe the association between OPCML and AXL expression in patients with CCA. Additionally, we also provide a promising concept of OPCML-based therapy for CCA which may improve clinical outcomes in the future.

Conflicts of Interest

The Authors declare no conflicts of interest.

Authors' Contributions

RK: Conceptualization, methodology, formal analysis, investigation, data curation, writing, visualization, and project administration; WW: methodology, formal analysis, and validation; JD: Conceptualization, methodology, and validation; CS: Conceptualization, methodology, and validation; TL: Conceptualization, methodology, validation, resources, writing and editing, supervision, and funding acquisition.

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