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Overexpression of polycomb repressive complex 2 key components EZH2/SUZ12/EED as an unfavorable prognostic marker in cholangiocarcinoma

Wiphawan Wasenang[a,b], Anucha Puapairoj[c], Chatri Settasatian[c], Siriporn Proungvitaya[a,d], Temduang Limpaiboon[a,d,*]

[a] Centre for Research and Development of Medical Diagnostic Laboratories, Faculty of Associated Medical Sciences, Khon Kaen University, Khon Kaen, 40002, Thailand
[b] Biomedical Sciences, Graduate School, Khon Kaen University, Khon Kaen, 40002, Thailand
[c] Department of Pathology, Faculty of Medicine, Khon Kaen University, Khon Kaen, 40002, Thailand
[d] Cholangiocarcinoma Research Institute, Faculty of Medicine, Khon Kaen University, Khon Kaen, 40002, Thailand

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ABSTRACT

BACKGROUND: Cholangiocarcinoma (CCA) is a fatal liver cancer arising from bile duct epithelium. Polycomb repressive complex 2 (PRC2) is a histone methyltransferase enzyme that catalyzes trimethylation of histone H3 on lysine 27, resulting transcriptional gene silencing. The key components of PRC2 are EZH2, SUZ12 and EED, which EZH2 is a catalytic subunit. The defect of individual PRC2 components has been shown to enhance carcinogenesis and cancer progression. The aim of this study was to determine the expression of individual PRC2 components and evaluate its association with clinicopathological data in CCA patients.

METHODS: The expression of PRC2 components including EZH2, SUZ12 and EED was determined by immunohistochemistry in 40 CCA tissue samples.

RESULTS: The expression of EZH2 and SUZ12 in CCA tissue was significantly higher than that in adjacent non-cancerous tissue ($P < 0.001$). The high cytoplasmic EZH2 expression was significantly associated with short overall survival in CCA ($P = 0.030$). Interestingly, a combined high nuclear and cytoplasmic expression of EZH2 was found to be a worse prognostic marker for overall survival ($P = 0.015$). Moreover, combined high expression of EZH2 and SUZ12/EED was also associated with short overall survival ($P < 0.05$).

CONCLUSIONS: Our findings suggest that overexpression of the PRC2 key components especially EZH2 in both nucleus and cytoplasm can be potentially used as a prognostic marker for CCA.

1. Introduction

Cholangiocarcinoma (CCA), although is an uncommon liver cancer originating from bile duct epithelial cells, is one of the top 10 most fatal cancers. The incidence of CCA is reportedly high in an endemic area of liver fluke, *Opisthorchis viverrini* infection, which has been approved as a causative agent of CCA by the World Health Organization [1–2]. The symptoms of CCA are obscure and being appeared when the disease progresses to advanced stage leading to the less opportunity to be cured. It has been reported that the median overall survival of CCA patients in Northeast Thailand was 4 months (95% CI, 3.3-4.7) and 2-year survival rate was only 8.1% (95% CI, 4.5-12.9) [3].

DNA methylation is an epigenetic event which has been widely studied in CCA over the past two decades whereas the study of histone methylation in this cancer is much less [4]. Histone methylation is the post-translational modification by enzymatic addition of methyl group to different lysine or arginine residues on histone tails leading to direct regulation of its target gene expression. Polycomb repressive complex 2 (PRC2) is a histone methyltransferase enzyme (HMT) that initially trimethylates histone H3 on lysine 27 (H3K27me3), resulting in target gene silencing. The key components of PRC2 are composed of Enhancer of zeste (EZH2), suppressor of zeste (SUZ12) and embryonic ectoderm development (EED), of which EZH2 is a core protein of PRC2 containing SET domain that acts as a catalytic site [5]. To fully exert of histone methyltransferase activity, EZH2 is indispensably required to interact with other non-catalytic subunits including SUZ12 and EED [6]. EED specifically binds to H3K27me3 enhancing allosteric activity and propagation of repressive histone marks [7], while SUZ12 is also required for stabilizing PRC2 activity [8]. The defect of individual PRC2 components has been demonstrated to reduce H3K27me3 level leading to

* Corresponding author at: Centre for Research and Development of Medical Diagnostic Laboratories, Faculty of Associated Medical Sciences, Khon Kaen University, Khon Kaen, 40002, Thailand.
E-mail address: temduang@kku.ac.th (T. Limpaiboon).

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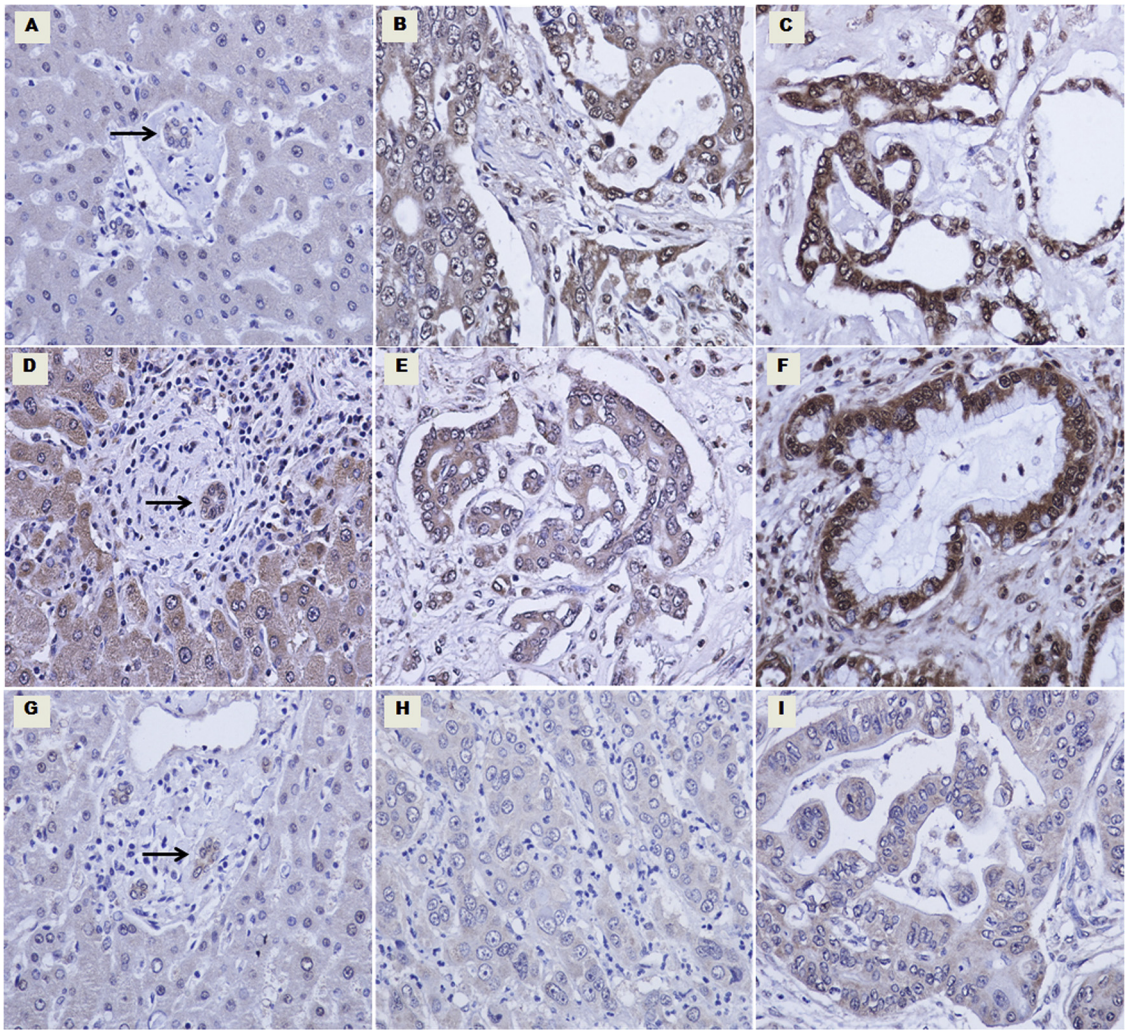

Fig. 1. Representative IHC staining of (A, B, C) EZH2, (D, E, F) SUZ12 and (G, H, I) EED expression in adjacent non-cancerous (A, D, G) and CCA tissues as low (B, E, H) and high (C, F, I) expression. The black arrows indicate bile duct cells of adjacent non-cancerous tissue. The immunohistochemical images were at 400x magnification.

derepression of PRC2 target genes that causes developmental abnormalities, carcinogenesis and cancer progression [9]. For instance, loss of SUZ12 leads to global loss of H3K27me3 level, non-integrity of PRC2 and instability of EZH2 [8,10]. EED I363M mutation has been shown to have an impairment of structural integrity that affected its binding ability to H3K27me3, resulting in the reduction of global H3K27me3 level and repressive histone marks [11]. Deletion of EZH2 also causes a depletion of global H3K27me3 impairing self-renewal, growth and differentiation in human embryonic stem cells [12]. Taken together, EZH2, SUZ12 and EED are crucial for PRC2 activity.

The association of EZH2 overexpression with poor prognosis and short overall survival has been demonstrated in several cancers such as glioma [13], multiple melanoma [14], chronic lymphocytic leukemia [15], prostate cancer [16], breast cancer [17] including CCA which promotion of cell proliferation and angiogenesis, and inhibition of apoptosis were also observed [18–21]. High expression of SUZ12 which promoted cell proliferation and metastasis in gastric cancer was significantly correlated with advanced stages, distant metastasis and short overall survival [22]. The overexpression of SUZ12 promoted cell proliferation in ovarian cancer which was also associated with shorter overall survival [23]. Collectively, these studies indicate the adverse effects of individual PRC2 key components when overexpressed on tumor progression and poor clinical outcomes. However, little is known regarding the status of all PRC2 key components in human cancer. Liu et al (2015) studied the expression of PRC2 key components including EZH2, SUZ12 and EED in patients with colorectal cancer. They found that elevated mRNA expression of all key components was correlated with poorer prognosis and shorter survival [24]. Cho et al (2018) studied the protein expression of PRC2 components, EZH2, SUZ12 and EED in patients with sarcoma subtypes and showed that combined high expression of all PRC2 was associated with shorter overall survival [25]. Here, we aimed to study the expression of individual PRC2 components including EZH2, SUZ12 and EED by immunohistochemistry in tissue samples of CCA patients. The association of their expression with clinicopathological parameters was also evaluated.

2. Materials and methods

2.1. Patients and samples

This study was approved by the Khon Kaen University Ethics Committee for Human Research (HE551066). Written informed consent was obtained from all patients. Resection specimens of CCA cases were randomly selected including 27 intrahepatic and 13 hilar types. The clinical data including age, gender, tumor size, tumor stage, histopathological grade, lymph node metastasis and survival time were kindly supplied by the Cholangiocarcinoma Research Institute, Khon

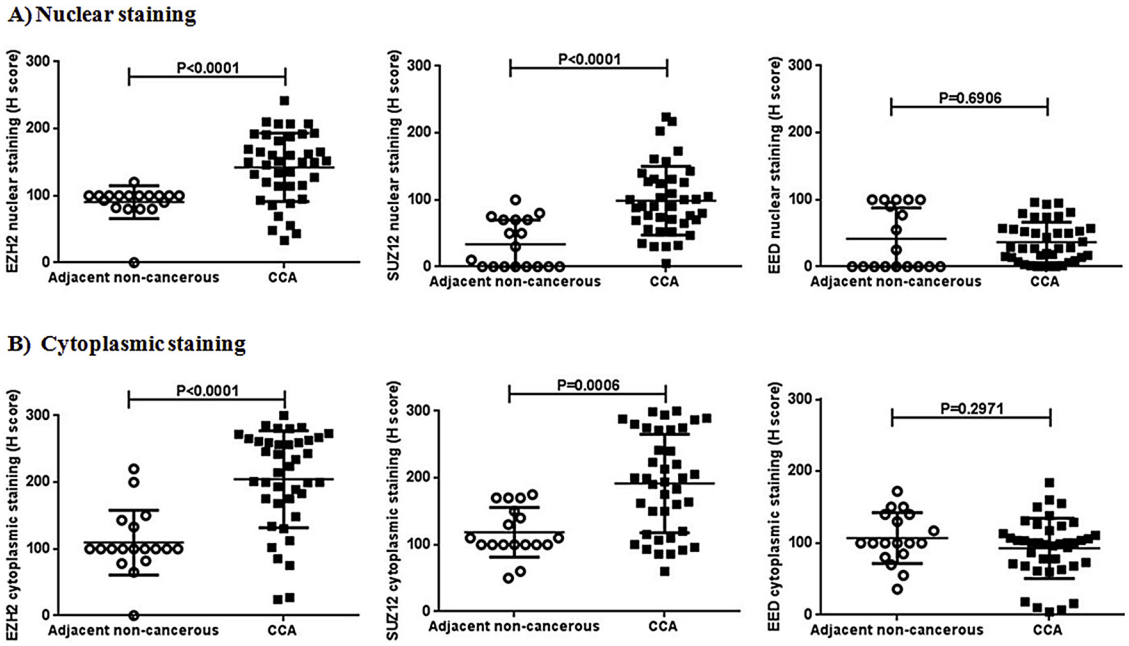

Fig. 2. Scatter plots of immunohistochemical scores (H-scores) of PRC2 components including EZH2, SUZ12 and EED between CCA (n = 40) and adjacent non-cancerous tissues (n = 18). (A) Nuclear expression and (B) Cytoplasmic expression of individual PRC2 components. The difference between two groups was analyzed by Mann-Whitney test.

Table 1
Clinical characteristics of enrolled CCA patients.

Clinical Characteristics	Cases
Total cases (n)	40
Intrahepatic type	27
Hilar type	13
Age (years, mean ± SD)	
58.53 ± 8.46	
Gender	
Male	25
Female	15
Tumor size	
≤ 7 cm	22
> 7 cm	18
Histopathological grade	
Well differentiated (WD)	13
Moderately differentiated (MD)	18
Poorly differentiated (PD)	9
Stage	
Early	7
Late	33
Lymph node metastasis	
Negative (N0)	14
Positive (N1)	20
No dissection (Nx)	6

Abbreviation: SD = Standard deviation.

Kaen University, Khon Kaen, Thailand.

2.2. Immunohistochemical (IHC) staining and IHC scoring

The paraffin-embedded tissue section of 4 μm thickness was deparaffinized and rehydrated. Antigen retrieval was performed by boiling slides in 0.01 M citrate buffer (pH 6.0). Endogenous peroxidases were blocked by treatment with 3% H_2O_2 for 1 h. Fetal bovine serum (20% V/V) was applied on the slides for blocking non-specific proteins for 2 h at room temperature. Primary antibody supplied by Biorbyt, UK (EZH2 antibody (orb229775), 1:400 dilution; SUZ12 antibody (orb69355), 1:2400 dilution; EED antibody (orb167285), 1:2000 dilution) was applied on the slides which then were incubated in a humidified box for 16 h at 4 °C. Antibody diluent was added on a slide as a negative control. The slides were washed with phosphate buffered saline with 0.05% tween 20, three times, 10 min for each wash before being incubated with secondary antibody (goat anti-rabbit for EED or anti-mouse for EZH2 and SUZ12, Envision™ System, DAKO Corporation, Carpinteria, CA) for 1 h at room temperature. The specific site of peroxidase activity was developed by using 3, 3'- diamino-benzidine tetrahydrochloride (DAKO Corporation). Finally, the slides were counterstained with Mayer's hematoxylin. The PRC2 expression in only bile duct cells of CCA and adjacent non-cancerous tissues was evaluated. The slides were examined and scored for individual PRC2 expression by two observers using semi-quantitative IHC scoring system [26]. H-scores were defined as the sum of the percentage of the stained cells multiplied by an ordinal value corresponding to the intensity level (0 = none, 1 = weak, 2 = moderate, 3 = strong), which ranged from 0 to 300. Localization of cytoplasmic and nuclear PRC2 staining was scored separately. Cutoff Finder [27] was used for determining optimal cut-off to dichotomize H-score of PRC2 as low and high expression for clinicopathological parameters analysis.

2.3. Statistical analysis

The statistical analysis was performed using SPSS version 16.0 for windows (SPSS Inc., Chicago, IL). The comparison of H-score of PRC2 expression between CCA and adjacent non-cancerous tissue was determined using Mann-Whitney test. The correlation between clinicopathological data and individual PRC2 expression in CCA was evaluated using Pearson χ^2 or Fisher's exact test. Overall survival curves were analyzed using Kaplan-Meier method and log-rank test. The Cox proportional hazards regression model was used for univariate and multivariate analysis. $P < 0.05$ was considered as statistically significant.

3. Results

3.1. Overexpression of PRC2 components in CCA tissue

To address the predominant localization of PRC2 expression, IHC

Table 2
Correlation between the nuclear expression of EZH2, SUZ12 and EED, and clinicopathological parameters in CCA.

Parameters	n (cases)	Nuclear EZH2 expression			Nuclear SUZ12 expression			Nuclear EED expression		
		Low n (%)	High n (%)	P-value	Low n (%)	High n (%)	P-value	Low n (%)	High n (%)	P-value
Total cases	40									
Age										
≤ 59 years	20	15 (75)	5 (25)	0.191	8 (40)	12 (60)	1.000	18 (90)	2 (10)	0.065
> 59 years	20	10 (50)	10 (50)		7 (35)	13 (65)		12 (60)	8 (40)	
Gender										
Male	25	15 (60)	10 (40)	0.746	10 (40)	15 (60)	0.746	16 (64)	9 (36)	0.026*
Female	15	10 (66.7)	5 (33.3)		5 (33.3)	10 (66.7)		14 (93.3)	1 (6.7)	
Tumor size										
≤ 7 cm	22	14 (63.6)	8 (36.4)	0.870	6 (27.3)	16 (72.7)	0.194	17 (77.3)	5 (22.7)	0.714
> 7 cm	18	11 (61.1)	7 (38.9)		9 (50)	9 (50)		13 (72.2)	5 (27.8)	
Histopathological grade										
WD	13	7 (53.8)	6 (46.2)	0.738	7 (53.8)	6 (46.2)	0.324	10 (76.9)	3 (23.1)	0.934
MD	18	12 (66.7)	6 (33.3)		5 (27.8)	13 (72.2)		13 (72.2)	5 (27.8)	
PD	9	6 (66.7)	3 (33.3)		3 (33.3)	6 (66.7)		7 (77.8)	2 (22.2)	
Stage										
Early (I, II)	7	7 (100)	0 (0)	0.006*	1 (14.3)	6 (85.7)	0.138	5 (71.4)	2 (28.6)	0.812
Late (III, IV)	33	18 (54.5)	15 (45.5)		14 (42.4)	19 (57.6)		25 (75.8)	8 (24.2)	
Lymph node metastasis										
Negative	14	12 (85.7)	2 (14.3)	0.026*	6 (42.9)	8 (57.1)	0.728	10 (71.4)	4 (28.6)	0.928
Positive	20	10 (50)	10 (50)		7 (35)	13 (65)		14 (70)	6 (30)	

Abbreviations: WD = well differentiated; MD = moderately differentiated; PD = poorly differentiated.
* Significantly different by the $\chi 2$ or Fisher's exact test.

Table 3
Correlation between the cytoplasmic expression of EZH2, SUZ12 and EED, and clinicopathological parameters in CCA.

Parameters	n (cases)	Cytoplasmic EZH2 expression			Cytoplasmic SUZ12 expression			Cytoplasmic EED expression		
		Low n (%)	High n (%)	P-value	Low n (%)	High n (%)	P-value	Low n (%)	High n (%)	P-value
Total cases	40									
Age										
≤ 59 years	20	13 (65)	7 (35)	1.000	7 (35)	13 (65)	1.000	16 (80)	4 (20)	0.716
> 59 years	20	12 (60)	8 (40)		6 (30)	14 (70)		14 (70)	6 (30)	
Gender										
Male	25	19 (76)	6 (24)	0.042*	9 (36)	16 (64)	0.539	21 (84)	4 (16)	0.094
Female	15	6 (40)	9 (60))		4 (26.7)	11 (73.3)		9 (60)	6 (40)	
Tumor size										
≤ 7 cm	22	13 (59.1)	9 (40.9)	0.747	5 (22.7)	17 (77.3)	0.185	16 (72.7)	6 (27.3)	0.713
> 7 cm	18	12 (66.7)	6 (33.3)		8 (44.4)	10 (55.6)		14 (77.8)	4 (22.2)	
Histopathological grade										
WD	13	7 (53.8)	6 (46.2)	0.499	4 (30.8)	9 (69.2)	0.668	9 (69.2)	4 (30.8)	0.846
MD	18	11 (61.1)	7 (38.9)		7 (38.9)	11 (61.1)		14 (77.8)	4 (22.2)	
PD	9	7 (77.8)	2 (22.2)		2 (22.2)	7 (77.8)		7 (77.8)	2 (22.2)	
Stage										
Early (I, II)	7	7 (100)	0 (0)	0.006*	0 (0)	7 (100)	0.074	7 (100)	0 (0)	0.034*
Late (III, IV)	33	18 (54.5)	15 (45.5)		13 (39.4)	20 (66.7)		23 (69.7)	10 (30.3)	
Lymph node metastasis										
Negative	14	11 (78.6)	3 (21.4)	0.150	5 (35.7)	9 (64.3)	0.727	12 (85.7)	2 (14.3)	0.166
Positive	20	11 (55)	9 (45)		6 (30)	14 (70)		13 (65)	7 (35)	

Abbreviations: WD = well differentiated; MD = moderately differentiated; PD = poorly differentiated.
* Significantly different by the $\chi 2$ or Fisher's exact test.

scoring of EZH2, SUZ12 and EED was assessed separately in nucleus and cytoplasm of CCA and adjacent non-cancerous tissue, and displayed as H-score. The representative IHC staining of EZH2, SUZ12 and EED in CCA tissue samples is shown in Fig. 1. EZH2 and SUZ12 were highly expressed in both nucleus and cytoplasm of CCA cells whereas low expression of EED was observed. By contrast, low expression of these three proteins was discerned in adjacent non-cancerous tissue (Fig. 1). As shown in Fig. 2, H-scores of both nuclear and cytoplasmic staining of EZH2 and SUZ12 in CCA were significantly higher than those in adjacent non-cancerous tissue while no significant difference was found in EED indicating high expression of PRC2, particularly EZH2 and SUZ12 in CCA.

3.2. The correlation of clinicopathological data with H-scores of EZH2, SUZ12 and EED in CCA patients

Clinicopathological characteristics of CCA patients are shown in Table 1. The H-scores of EZH2, SUZ12 and EED in both nucleus and cytoplasm were evaluated for cut-off values using the Cutoff Finder [27], which were 161 and 251, 76.5 and 155, and 56.5 and 115, respectively, and were used to divide CCA patients into two groups as low and high expression. The correlation between the expression levels of EZH2, SUZ12 and EED in both nucleus and cytoplasm, and clinicopathological parameters including age, gender, tumor size, histopathological grade, stage and lymph node metastasis of CCA was

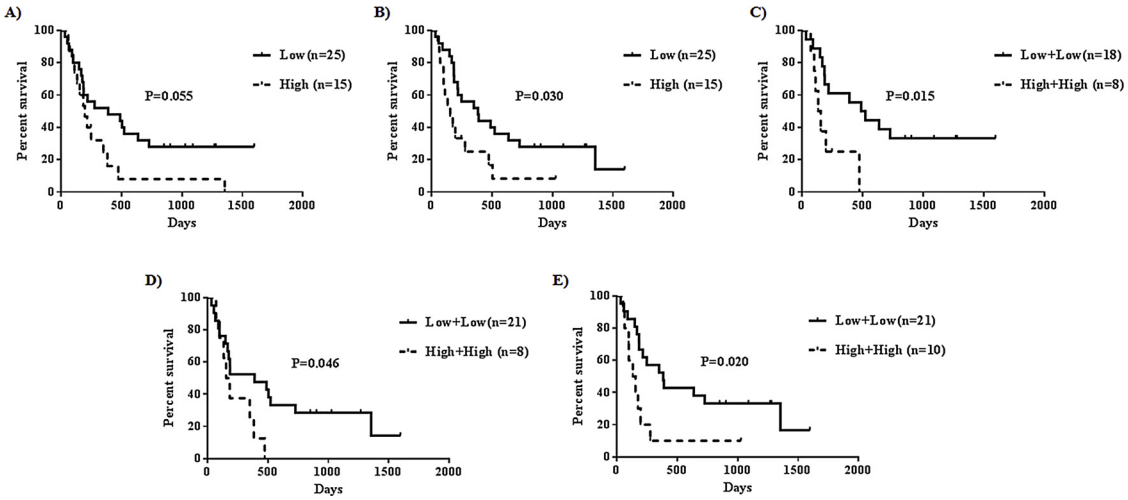

Fig. 3. Kaplan-Meier curves of the expression of key PRC2 components in CCA patients (A) nuclear EZH2 (B) cytoplasmic EZH2 (C) combination of nuclear and cytoplasmic EZH2 (D) combination of nuclear EZH2 and SUZ12/EED (E) combination of cytoplasmic EZH2 and SUZ12/EED.

determined (Tables 2 and 3). The results showed that the low nuclear EZH2 expression was significantly correlated with early stage and negative lymph node metastasis (P = 0.006 and P = 0.026, respectively). Besides, the low cytoplasmic expression of EZH2 and EED was also significantly correlated with early stage (P = 0.006 and P = 0.034, respectively). There was no correlation between SUZ12 expression and clinicopathological parameters (P > 0.05).

3.3. Overexpression of PRC2 is associated with shorter overall survival in CCA patients

The correlation between the expression levels of EZH2, SUZ12 and EED, and overall survival was determined by Kaplan Meier analysis. We found that CCA patients with high cytoplasmic EZH2 expression had significantly shorter overall survival than those with low expression (P = 0.030) (Fig. 3B). However, patients with high nuclear EZH2 expression tended to have poor prognosis (P = 0.055) (Fig. 3A). Interestingly, the combination of high expression of nuclear and cytoplasmic EZH2 showed more significant association with short overall survival when compared to combined low expression (P = 0.015) (Fig. 3C). The expression levels of SUZ12 and EED in both nucleus and cytoplasm were not correlated with overall survival (P > 0.05). However, when nuclear or cytoplasmic EZH2 expression was combined with nuclear or cytoplasmic expression of SUZ12 and/or EED, it was found that patients with the combined high expression of nuclear EZH2 and SUZ12 and/or EED had poorer prognosis than those with combined low expression (P = 0.046) (Fig. 3D). The short survival was also observed in patients with combined high expression of cytoplasmic EZH2 and SUZ12 and/or EED (P = 0.020) (Fig. 3E). Our findings indicate the association of high expression of combined EZH2 in both nucleus and cytoplasm with unfavorable prognosis of CCA patients which can be used as a potential prognostic marker for CCA. Notably, the importance of PRC2 localization in both nuclear and cytoplasmic staining should be concerned in order to get more information regarding CCA progression. Furthermore, the multivariate Cox regression analysis after adjusting for gender demonstrated that lymph node metastasis was an independent prognostic marker for CCA with hazard ratio of 2.980 (95%CI, 1.133-7.839) as shown in Table 4.

4. Discussion

PRC2 plays an important role in embryonic development and differentiation by regulating the expression of developmental genes especially key transcription factors [28]. PRC2 trimethylates H3K27 resulting in target gene repression which is generally observed in pluripotent to differentiated cells by which the expression of PRC2 in lineage-specific transcription depends on individual cell fates [28]. Although high expression of PRC2 components was found in CCA compared to adjacent non-cancerous tissue, no significant difference was observed in EED. The immunohistochemical images derived from the Human Protein Atlas (www.proteinatlas.org) exhibit no expression of EZH2, SUZ12 and EED in normal bile ducts but increased expression in CCA [29]. Sasaki et al (2014) showed EZH2 expression in cholangiolocellular carcinoma but not in bile duct adenomas and ductular reactions [21]. Our study could detect the expression of individual PRC2 components in adjacent non-cancerous tissue suggesting that molecular alterations inside the cells may occur while cell morphology remains unchanged. PRC2 expression was observed in both nucleus and cytoplasm of CCA tissue. PRC2 function is predominantly located in nucleus but can also be observed in cytoplasm by regulating cellular processes in mammalian cells [30–32]. In this study, the combined expression of nuclear and cytoplasmic EZH2 in CCA tissue has increased the possibility to find the association with poor overall survival indicating the important role of PRC2 components not only in nucleus but also in cytoplasm which may contribute to tumor progression.

The overexpression of EZH2 observed in our study was associated with short overall survival which agreed with the previous studies in CCA and other cancers [13–19]. Lymph node metastasis as an independent prognostic factor for CCA patients was observed in this study which was similar to the previous report [33]. Although increased SUZ12 or EED expression was not significantly associated with overall survival, a significant association with unfavorable prognosis was observed in a combination of high EZH2 and high SUZ12 and/or EED expression. Our findings suggest that a significant association of PRC2 components with overall survival was dependent on EZH2 expression. EZH2 is a catalytic subunit of PRC2 which exhibits methyltransferase activity by trimethylation of H3K27. However, its function also indispensably requires SUZ12 and EED to maintain the integrity of PRC2 complex which mediates H3K27me3 repressive marks [34–35]. Our finding on a combination of high EZH2 and high SUZ12 and/or EED expression suggests the important role of PRC2 complex activity contributing to CCA progression. Previous reports showed that the expression of EZH2 was related to H3K27me3 level in gastric cancer [36] and lymphoma [37]. Moreover, Cho et al (2018) found the relation of PRC2 key components, EZH2, SUZ12 and EED, and H3K27me3 in sarcoma subtypes [25]. However, our study did not perform H3K27me3 immunostaining which may reflect the function of PRC2 in CCA. Further study should be conducted to get insight into the association of

Table 4
The Cox proportional hazards regression analysis of clinicopathological parameters and the expression of EZH2, SUZ12 and EED in CCA.

Parameters (n)	Univariate		Multivariate[a]	
	HR (95%CI)	P-value	HR (95%CI)	P-value
Age, years				
< 59 (20)	1	0.717	-	-
≥ 59 (20)	0.879 (0.439-1.762)			
Gender				
Male (25)	1	0.054	-	-
Female (15)	0.486 (0.439-1.762)			
Tumor size				
≤ 7cm (22)	1	0.134	-	-
> 7cm (18)	1.720 (0.847-3.495)			
Histopathological grading				
WD + MD (31)	1	0.142	-	-
PD (9)	1.829 (0.816-4.099)			
Stage				
I, II (7)	1	0.129	-	-
III, IV (33)	2.292 (0.786-6.687)			
Lymph node metastasis				
Negative (14)	1	0.005*	2.980 (1.133-7.839)	0.027*
Positive (20)	3.723 (1.490-9.301)			
IHC staining				
EZH2 nuclear				
Low expression (25)	1	0.059	-	-
High expression (15)	2.022 (0.974-4.200)			
EZH2 cytoplasmic				
Low expression (25)	1			
High expression (15)	2.210 (1.063-4.593)	0.034*	2.212 (0.93-5.261)	0.072
SUZ12 nuclear				
Low expression (15)	1	0.09	-	-
High expression (25)	0.538 (0.263-1.101)			
SUZ12 cytoplasmic				
Low expression (13)	1	0.271	-	-
High expression (27)	0.663 (0.318-1.378)			
EED nuclear				
Low expression (30)	1	0.270	-	-
High expression (10)	0.622 (0.268-1.445)			
EED cytoplasmic				
Low expression (30)	1	0.322	-	-
High expression (10)	1.484 (0.679-3.240)			

Abbreviations: HR = hazard ratio; CI = confidence interval; WD = well differentiated; MD = moderately differentiated; PD = poorly differentiated.
[a] The multivariate model is adjusted for gender.
* Statistically significant.

PRC2 complex and H3K27me3.

In summary, we have shown that high EZH2 expression or in combination with SUZ12 and/or EED expression is associated with poorer overall survival that could be used as a prognostic marker for CCA. The prognostic value of EZH2/SUZ12/EED in a large sample size should be warranted before implementation for all CCA patients. Moreover, PRC2 overexpression may be used as a predictive marker for CCA treatment. Histone methyltransferase inhibitors would be suggested as a therapeutic drug for treatment of CCA patients which may improve their clinical outcomes.

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