ORIGINAL ARTICLE

Clinicopathological Features of Epstein-Barr Virus-associated Intrahepatic Cholangiocarcinoma

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Background: Epstein-Barr virus (EBV) was found to be associated with many cancers. Only a few cases of EBV-associated cholangiocarcinoma were reported and most of them were lymphoepithelioma-like tumors. The objective of this study was to determine the prevalence of EBV in intrahepatic cholangiocarcinoma in the Thai population.

Materials and Methods: A total of 40 cholangiocarcinoma tumors were retrieved by simple random sampling. EBV status was assessed by EBV-encoded small RNA (EBER) in situ hybridization from formalin-fixed paraffin-embedded (FFPE) tumor samples. The association between EBV status and clinicopathological characteristics was analyzed using Fisher's exact test, and survival analysis was done using the Kaplan-Meier method.

Results: Out of 40 intrahepatic cholangiocarcinoma tumor samples, 3 (7.5%) were positive for EBV by EBER in situ hybridization. The median age of EBV-positive patients was lower than the EBV-negative group (52 vs. 64 years). All three EBV-positive intrahepatic cholangiocarcinoma cases were presented in stage I (T1N0M0). Two patients were well-differentiated adenocarcinoma and 1 was mucinous cystadenocarcinoma. There was a trend toward better survival was seen in EBV-positive patients (p=0.085).

Conclusion: EBV-associated intrahepatic cholangiocarcinoma is rare, only 7.5%, even in the endemic area.

Keywords: Biliary duct cancer; Intrahepatic cholangiocarcinoma; Epstein-Barr virus (EBV); EBV-associated cancer; EBER

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Liver flukes (*Clonorchis sinensis* and *Opisthorchis viverrini*), primary sclerosing cholangitis, and biliary system malformation are known risk factors for cholangiocarcinoma (CCA). Hepatitis B and C virus, as well as nitrosamine toxins, have been identified as additional risk factors for CCA[1-3]. In a few CCA cases, the Epstein-Barr virus (EBV) has also been reported[4-6].

EBV-associated malignancies accounted for approximately 5.5 percent of all infection-related cancers and 1.9 percent of the global cancer burden[7,8]. Nasopharyngeal carcinoma (NPC), Burkitt lymphoma, Hodgkin lymphoma, and post-transplant lymphoproliferative disease are all linked to EBV[8-11]. Furthermore, recent reports have revealed a relationship between EBV and stomach cancer. EBV was found in 8.8% of the 9,738 gastric adenocarcinoma patients in the meta-analysis, and it has a distinct molecular and pathological feature that may respond to immunotherapy[12].

The first case of EBV was discovered in a lymphoepithelioma-like (LEL) cholangiocarcinoma, a rare variant of intrahepatic CCA[13]. Large, syncytial cells with a prominent lymphocytic infiltrate characterize LEL carcinoma. The distinct pathology of LEL carcinoma is related to EBV and has also been reported in several cancers. EBV was found in 3.3 percent of intrahepatic cholangiocarcinoma patients in a Chinese study. Among these EBV-positive patients, 72.7% were lymphoepithelioma-like CCA[14].

Thailand is an endemic area for both EBV infection and cholangiocarcinoma. Thai children are exposed to

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EBV from a very early age in life[15]. Nonetheless, little is known about the Epstein-Barr virus in patients with cholangiocarcinoma. Hence, the authors investigated the prevalence of EBV-associated cholangiocarcinoma (EBVaCCA) in the Thai population and its clinical features.

Materials and Methods

Patients and samples

The present study was a pilot study and included 40 samples of cholangiocarcinoma patients who underwent hepatectomy at Srinagarind hospital between 2014 and 2018. The patients were excluded if the tissue was inadequate. Formalin-fixed paraffin-embedded blocks were retrieved and re-reviewed. Age, sex, tumor characteristics, TNM staging, and clinical follow-up data were collected retrospectively as baseline clinicopathological parameters.

EBV-encoded small RNA (EBER) in situ hybridization (ISH)

Resected specimens were fixed in 10% neutral buffered formalin, dehydrated, embedded in paraffin, and thin sectioned by routine histology methods. To examine the EBV in specimens, the slide was selected, deparaffinized, buffered, applied protease, and EBER-ISH was performed using the XT INFORM Probes iVIEW Blue V3 procedure (Ventana Medical Systems, Inc., Tucson, AZ, USA). Slides were processed by an automated stainer (Benchmark XT; Ventana Medical Systems, Inc.) and stained using the ISH iView Blue Detection Kit (Ventana Medical Systems, Inc.) with Red stain II (Ventana Medical Systems, Inc.) being used as counterstain. Specimens were considered EBER-positive if any expression of EBER was observed in the specimens. All pathological specimens were categorized into specific histologic subtypes of intrahepatic cholangiocarcinoma.

Statistical analysis

Descriptive statistics for baseline data were presented as percentages, mean, and standard deviation. If the distribution of these data was not normal, the median and interquartile range would then be used. The Fisher exact tests were used to determine the difference between the two groups.

Overall survival (OS) was defined as the time from surgery until the date of death or the end of follow-up. Survival analysis was performed using the Kaplan-Meier method and the log-rank test. A p-value less than 0.05 was statistically significant in all tests. All data analyses were carried out using STATA software (StataCorp LP, College Station, TX, USA).

Ethical approval was provided by the Khon Kaen University Ethics Committee for Human Research based on the Declaration of Helsinki (Number HE621031).

Table 1. Baseline clinical characteristics, values expressed as n (%)

Characteristics	n=40 (%)
Age (years), median (range)	63 (40 to 82)
Male	26 (65)
Female	14 (35)
T1	15 (37.5)
T2	12 (30)
T3	4 (10)
T4	9 (22.5)
N0	32 (80)
N1	8 (20)
HBV +ve	1 (2.5)
HBV -ve	38 (95)
HBV unknown	1 (2.5)
HCV +ve	1 (2.5)
HCV -ve	38 (95)
HCV unknown	1 (2.5)
CA 19-9 (IU/mL), median (IQR)	11.3 (0.73, 9)
CEA (ng/mL), median (IQR)	3.3 (2.4, 6.7)
Tumour size (cm), median (IQR)	4.75 (2.6, 7.25)
Right side	28 (70)
Left side	12 (30)
Histology	
Well-differentiated adenocarcinoma	21 (52.5)
Adenosquamous carcinoma	2 (5)
Invasive papillary carcinoma	16 (40)
Mucinous cystadenocarcinoma	1 (2.5)

HBV=hepatitis B virus; HCV=hepatitis C virus; CA 19-9=carbohydrate antigen 19-9; CEA=carcinoembryonic antigen

Results

A total of 40 patients were included, of which 26 were male and 14 were female. The mean age was 63.4 years. Most of the patients were in the T1 and T2 stage (37.5% vs. 30%, respectively) as shown in Table 1 with the median size of the tumor of 4.75 cm. Twenty-one patients (52.5%) were well-differentiated adenocarcinoma, 16 (40%) were invasive papillary carcinoma, 2 (5%) were adenosquamous carcinoma, and 1 (2.5%) was mucinous cystadenocarcinoma. In terms of comorbid viral hepatitis, 1 (2.5%) had HBV infection and 1 (2.5%) had HCV infection. No liver fluke was detected in all tumor samples.

EBV positive and clinicopathological features

Three of the 40 tissue samples (7.5%) were EBV-positive by EBER in situ hybridization.

Associated factors and EBV status are summarized in Table 2. EBV-positive patients were younger than the negative group (median 52 vs. 64 years). The tumor size difference between the EBV-positive (3.6 cm) and EBV-negative group (4.8 cm) was not significant. The

Table 2. EBV status and associated factors

	EBV-positive; n (%), n=3	EBV-negative; n (%), n=37	p-value
Age (years)			0.05
Median (Range)	52 (50 to 62)	64 (43 to 82)	
Mean (SD)	54.7 (6.4)	64.1 (8.0)	
Sex			0.95
Male	2 (66.7)	24 (65)	
Female	1 (33.3)	13 (35)	
CA 19-9 (IU/mL), Median (IQR)	32.8 (7.5, 73.9)	8.8 (0, 118)	0.46
CEA (ng/mL), Median (IQR)	2.7 (2.1, 15.2)	3.4 (2.5, 6.7)	0.88
Tumour size (cm), Median (IQR)	3.6 (2.5, 11.1)	4.8 (2.7, 7.0)	0.79
Stage			0.05
1	3 (100)	12 (32.4)	
2	0	9 (24.3)	
3	0	16 (43.2)	
HBV +ve	1 (33.3%)	0	0.07
HCV +ve	0	1 (2.5)	1.0
Histology			1.0
Well-diff adenocarcinoma	2 (66.7)	19 (51.4)	
Adenosquamous carcinoma	0	2 (5.4)	
Invasive papillary carcinoma	0	16 (43.2)	
Mucinous cystadenocarcinoma	1 (33.3)	0	

EBV=Epstein-Barr virus; HBV=hepatitis B virus; HCV=hepatitis C virus; CA 19-9=carbohydrate antigen 19-9; CEA=carcinoembryonic antigen

Table 3. Clinical characteristics of EBV-associated cholangiocarcinoma (EBVaCCA)

Case	Age (years)	Sex	Site	Size (cm)	HBV	HCV	Histology	CA 19-9 (IU/mL)	Status
1	50	M	Rt.	3.6	+	-	Well-diff. Adenocarcinoma	32.8	Alive
2	52	F	Rt.	11.1	-	-	Mucinous Cystadenocarcinoma	73.9	alive
3	62	M	Lt.	2.5	-	-	Well-diff. Adenocarcinoma	7.5	alive

HBV=hepatitis B virus; HCV=hepatitis C virus; CA 19-9=carbohydrate antigen 19-9; M=male; F=female

EBV-positive group had a higher median level of CA19-9, although it was not statistically significant.

EBV positive patients

Table 3 summarizes the characteristics of all the EBV-positive patients. Notably, all of the EBVaCCA cases were in stage T1N0M0. The histopathology results were well-differentiated adenocarcinoma in two patients (66.7%) and mucinous cystadenocarcinoma in one patient. All three EBV-positive patients had no lymphoepithelioma-like features. The histopathological findings of the EBV-positive samples are shown in Figure 1.

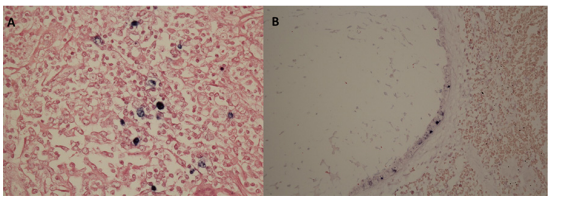

Figure 1. Histopathology of EBER-positive intrahepatic CCAs. The EBER-ISH positive case of the nuclear staining in varying intensity of the tumor cells (A, 40x) and the dysplastic large bile duct (B, 40x).

EBV and survival data

A trend towards a longer survival was observed in EBVaCCA patients. The median OS for the EBV-positive group was not reached, while the median OS for the EBV-negative group was 30.4 months (19.9 months - NR); the log-rank test p-value was 0.085 (Figure 2).

Fifteen patients in this cohort were presented in stage 1 (T1N0M0). Among these, 3 patients were EBV-positive, and 12 patients were EBV-negative. Figure 3 shows that even among T1N0M0 stage, there was a trend towards better overall survival in EBVaCCA patients (p=0.24).

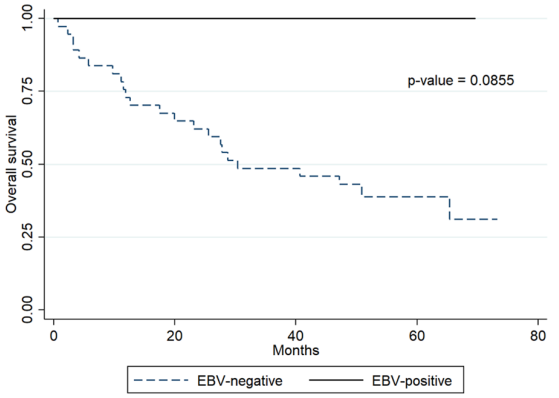

Figure 2. Overall Survival comparing the EBV-positive vs. EBV-negative intrahepatic CCAs.

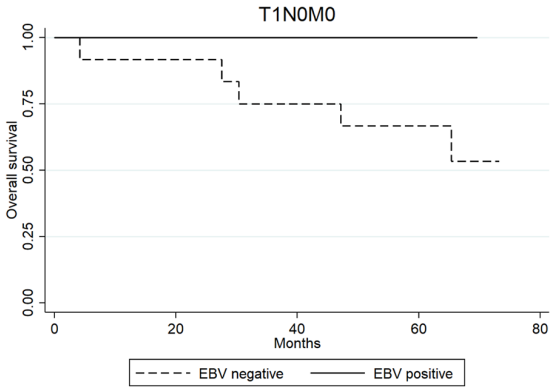

Figure 3. Overall survival in EBV-positive vs. EBV-negative T1N0M0 intrahepatic CCAs.

Discussion

The association between cholangiocarcinoma and EBV is still unclear even in the endemic area. The present study demonstrated the prevalence of EBVaCCA of 7.5 percent in northeast Thailand, which was comparable to 6.6 to 6.8% from southern China, where EBV is highly endemic[5,6]. The number is slightly lower when compared with the prevalence of EBV-positive diffuse large B-cell lymphoma from the same region of 11.1 percent[16].

No distinct pathological characteristic was identified in EBVaCCA in the present study. All EBVaCCA patients in this cohort had conventional adenocarcinoma with no LEL feature. Patients with EBVaCCA were younger than those with EBV-negative tumors, which matches findings other EBV-associated cancer studies[17,18].

In the present study, EBVaCCA patients were presented at the early clinical stage and there was a trend toward better survival when compared to EBV-negative patients. The findings are similar to data from the large international pooled analysis of over 4,500 gastric cancer patients, which revealed the correlation between EBV and an earlier clinical stage and a longer survival time[19]. The better 2- and 5-year overall survival was also demonstrated in EBV positive CCA patients compared to negative cases in one case series[4]. Although, there was no difference in overall survival between the EBV-positive group and negative group in the large study from China, those with the LEL subtype significantly related to favorable prognostic outcomes[5].

Currently, there is no evidence that EBV directly induces biliary tract carcinogenesis; however, it is believed that the monoclonal proliferation of a single cell persistently infected with EBV alters the tumor microenvironment and results in carcinoma[11,20-22]. Whether there is a relationship between EBV and Opisthorchiasis is still unknown[23]. No liver fluke was identified in EBVaCCA in the present study. EBVaCCA was associated with the higher HBV infection rate in a study from China[5]; however, in the present study, there was only one patient with HBV infection.

The main limitation of the present study was the small sample size with only intrahepatic CCA. Furthermore, comprehensive molecular profiling related to EBV infection, and its tumor microenvironment was not performed. A larger study in the endemic area is warranted. The EBV detection technique used in the present study was only EBER-ISH with no parallel method. Moreover, there is no comparison between the prevalence of EBV in matched healthy population compared to cancer patients.

Conclusion

The prevalence of EBVaCCA was 7.5% and the subtype is conventional well-differentiated adenocarcinoma.

What is already known on this topic?

Epstein-barr virus is associated with pathogenesis of nasopharyngeal and gastric cancer. Few reports of EBV-associated cholangiocarcinoma and the pathology is mostly lymphothelioma-like variant.

What this study adds?

In the endemic area of cholangiocarcinoma, the prevalence of EBVaCCA was low and patients were presented in the early stage.

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Conflicts of interest

The authors declare no conflict of interest.

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