The Immune Microenvironment Features and Response to Immunotherapy in EBV-associated Lymphoepithelioma-like Cholangiocarcinoma

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Research Article

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Abstract

Background & Aims Limited data are available for tumor immune microenvironment (TIME) in Epstein-Barr virus (EBV)-associated lymphoepithelioma-like cholangiocarcinoma (EBV-LELCC), a rare subtype of intrahepatic cholangiocarcinoma (IHCC). We aimed to investigate TIME features in EBV-LELCC and the correlation between the components of TIME and the clinical outcomes.

Methods Tumor tissues from Five EBV-LELCC cases confirmed through EBER in situ hybridization and five stage-matched conventional IHCC (non-EBV IHCC) cases were collected. These samples were used to evaluate genetic alterations, TIME composition, and PD-L1 expression through ion AmpliSeq comprehensive cancer panel, PanCancer immune profiling panel, immunohistochemistry, and immunofluorescence staining. The correlation between clinical outcomes and TIME components was analyzed in the two EBV-LELCC cases receiving anti-PD-1 treatment.

Results The genetic mutations identified in EBV-LELCC were *BARD1*, *CD19*, *CD79B*, *EPHA5*, *KDM5A*, *MUC6*, *MUC16*, *PTEN*, *RECQL4*, *TET1*, and *TNFAIP3*. PD-L1 was highly expressed in tumor-infiltrating immune cells, especially the T cells and macrophages. The TIME of EBV-LELCC displayed abundant immune cell infiltration with a stronger adaptive immune response. Increased Th1 cells, NK CD56_{dim} cells, and M1 macrophages, decreased M2 macrophages, exhausted CD8 T cell infiltration, and increased T cell activation signatures in TIME were associated with longer survival. Two patients with metastatic EBV-LELCC had good disease control after anti-PD-1 antibody treatment. A significantly larger TIME component made EBV-LELCCs more sensitive to immune checkpoint blockade (ICB).

Conclusion A better understanding of the composition of TIME in EBV-LELCC is critical for predicting the clinical outcomes of ICB treatment.

Lay Summary

Epstein-Barr virus-associated lymphoepithelioma-like cholangiocarcinoma (EBV-LELCC) is a unique variant of intrahepatic cholangiocarcinoma with dense infiltration of immune cells, different genetic backgrounds, and tumor immune microenvironment (TIME). The components of TIME may predict clinical outcome in EBV-LELCC and can potentially be exploited to enhance the efficacy of immunotherapy. Patients with EBV-LELCC are good candidates for the clinical benefit from immunotherapy.

Introduction

Intrahepatic cholangiocarcinoma (IHCC) is the second most common primary carcinoma of the liver and the incidence of IHCC has increased globally, with the highest incidence in East Asia [1]. The etiological factors of IHCC include biliary stone-related cholestasis and chronic viral infections, such as hepatitis B and C, which initiate the basis for chronic biliary inflammation and damage [2]. Different carcinogenetic pathways result in different histological findings in which chronic hepatitis or cirrhosis induces mass-

forming peripheral small duct type IHCC, while perihilar large duct type and intraductal growth type IHCCs are highly related to chronic biliary inflammation [3]. The different morphologies are correlated with different molecular features, including genetic alternations and tumor immune microenvironment (TIME) [4].

Lymphoepithelioma-like cholangiocarcinoma (LELCC), a rare variant of IHCC, is characterized by a unique morphology with syncytial sheets of carcinoma cells and dense tumor-infiltrating lymphocytes (TILs), with no obvious desmoplasia throughout the tumor [5]. The majority of LELCC cases are from Asian populations and mostly associated with Epstein-Barr virus (EBV) infection, within the range of 55% and 73.1% [6, 7]. EBV is an oncogenic herpes virus and has been increasingly identified in diverse malignancies including well-known EBV-associated nasopharyngeal carcinoma and gastric cancer (GC) [5, 8]. In EBV-IHCC, EBV circular viral genomes are present in tumor cells, but not in the adjacent non-neoplastic epithelium of the bile duct [9].

Programmed death-ligand 1 (PD-L1) is a ligand targeting programmed cell death protein 1 (PD-1), which is present in both tumor cells and TILs in EBV-LELCC with increased expression of the former [9, 10]. Chronic inflammatory environments resulting from virus-associated malignancies may promote PD-L1 upregulation and alter the TIME [11]. Herein, we presented the composition of immune cell infiltration in patients with EBV-LELCC and identified the correlation between the components of TIME and clinical outcomes in two patients who received immunotherapy alone or in combination with chemotherapy.

Material And Methods

Patients and data collection

The study was approved by National Cheng-Kung University Hospital (B-ER-106-224) and Taipei Veterans General Hospital (2015-03-005BC). Five patients that underwent curative surgery with pathological diagnosis of EBV-LELCC during October 2016 and January 2018 were enrolled. For comparison, five cases of conventional IHCC (non-EBV IHCC) were selected randomly by matching with early or late stages, diagnosed during July 2013 and January 2018. Formalin-fixed, paraffin-embedded (FFPE) tumor tissue sections were collected for the following analysis. Electronic medical records (EMRs) were reviewed to obtain information about the baseline characteristics of age, initial tumor size, stage, history of hepatitis B and C, and the date of last follow-up visit. Overall survival (OS) was defined as the duration from the time of pathological diagnosis to the date of death, and disease-free survival (DFS) was defined as the time of cancer recurrence, second cancer, or death from any cause obtained from the EMRs.

EBV-encoded RNA in situ hybridization

To detect early RNA transcripts in 4 µm-thick FFPE sections, INFORM EBER Probes (Catalog number 800-2842, Ventana, Tucson, AZ, USA) and VENTANA ISH iView Blue Detection Kit (Catalog number 800-092, Ventana, Tucson, AZ, USA) were used. The slides were counterstained with nuclear fast red solution (Merck KGaA, Germany), with EBV-positive nasopharyngeal carcinoma as positive control.

Sample processing and Ion AmpliSeq Comprehensive Cancer Panel (IACCP)

Manual microdissection was performed to isolate tumor tissues from FFPE sections in five cases of EBV-LELCC for DNA extraction using QIAmp DNA FFPE Tissue Kit (Qiagen, Hilden, Germany). DNA was amplified using Ion AmpliSeq Comprehensive Cancer Panel (IACCP, Life Technologies, Carlsbad, CA, USA) consisting of 409 cancer-related genes. The quality and quantity of extracted DNA and the amplified library were determined using fragment analyzer (Advanced Analytical Technologies, Inc.) and Qubit (Invitrogen, Carlsbad, CA, USA). Sequencing was conducted on ion proton sequencer using an ion P1 chip (Life Technologies) according to the manufacturer's protocol, with a mean sequencing depth of >800x.

RNA extraction, nCounter® PanCancer Immune Profiling Panel (PIPP), and quantitative real time polymerase chain reaction (qPCR)

RNA was extracted from the dissected tumor tissues of five non-EBV IHCC and five EBV-LELCCs using RNeasy FFPE Kits (Qiagen) and applied to the NanoString PIPP, a panel assessing expression of 770 genes, according to the manufacturer's instructions. Genes defined as cell type-specific were used to calculate immune cell type scores. The qPCR assay was applied for PDCD1 and CD274 mRNA level detection. GAPDH was used as an internal control.

Hematoxylin and eosin (H&E) staining, immunohistochemistry (IHC) staining, and PD-L1 scoring

FFPE tissues of EBV-LELCC were cut into 5-mm-thick sections and stained using H&E or primary antibodies including PD-L1, CK7, CD20, CD56, CD68, CD3, CD4, and CD8 by IHC analysis. PD-L1 expression was determined using tumor positive score (TPS) as follows: the number of positive tumor cells divided by the total number of viable tumor cells multiplied by 100% as well as using combined positive score (CPS) as follows: the number of positive tumor cells, and mononuclear cells divided by the total number of viable tumor cells multiplied by 100.

Immunofluorescence (IF) staining

Human EBV-LELCC sections were deparaffinized and rehydrated. Antigen retrieval was performed using target retrieval buffer solution (Dako, S1699). Following endogenous peroxidase blocking and incubation in superbloc blocking buffer (Thermo, 37515) for 60 min, the sections were incubated with primary antibodies against PD-L1 (Abcam, ab205921; 1:50 dilution), CD3 (Genetex, GTX16669; 1:50 dilution), and CD68 (Dako, M0814; 1:500 dilution) at 4°C overnight followed by incubation with secondary antibodies at 25°C for 30 min. Cell nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI). The images of the sections were obtained through FACS-like Tissue Cytometry (Tissue Gnostics, Vienna, Austria).

Gene set enrichment analysis (GSEA)

GSEA 4.1.0 (<https://www.gsea-msigdb.org/gsea/downloads.jsp>, accessed July 30, 2020, Broad Institute) was used to identify enriched molecular signatures based on experimentally validated gene sets deposited in Molecular Signature Database (MSigDB, hallmark gene sets: h.all.v7.1.symbols.gmt, biocarta gene sets: c2.cp.biocarta.v7.1.symbols.gmt, Kyoto Encyclopedia of Genes and Genomes (KEGG) gene sets: c2.cp.kegg.v7.1.symbols.gmt, reactome gene set: c2.cp.reactome.v7.1.symbols.gmt, Gene Ontology (GO) gene sets: c5.all.v7.1.symbols.gmt, oncogenic gene sets: c6.all.v7.1.symbols.gmt, and immunologic gene sets: c7.all.v7.1.symbols.gmt). Only gene sets with a P value of <0.05 were considered statistically significant.

Statistical analysis

All analyses were performed using GraphPad Prism version 5.0 (GraphPad Software, La Jolla, CA). Data of all the experiments are expressed as the mean $\pm$ standard error. Statistical significance was set at $P < 0.05$.

For further details regarding the materials and technique used, please refer to the supplementary information.

Results

Clinical data and pathological findings

The clinical characteristics and survival status of non-EBV IHCC and EBV-LELCC are summarized in Table 1. For the five EBV-LELCC cases, the mean age was 61.4 years (range, 41–75 years), which was comparable to non-EBV IHCC cases (median age of 60.2 years). All EBV-LELCC patients including three with pathological stage I and two with stage III/IV, according to the American Joint Committee on Cancer 7th edition, underwent surgery. Four EBV-LELCC patients still survived on data cut-off. Only one patient suffered from chronic viral hepatitis B infection. The histological findings of lymphoepithelioma pattern showed sheets of glandular tumor cells with prominent nucleoli, a fair amount of cytoplasm, and dense lymphoplasmic cell infiltration (Fig. 1a). The tumor cells, but not the background inflammatory cells were positive for EBER *in situ* hybridization in all EBV-LELCC cases.

Table 1
Clinical characteristics and survival time of non-EBV IHCC and EBV-LELCC.

Case	Age (year)	Gender	Tumor size (cm)	Stage [#]	HBV	HCV	Survival after surgery (status)
non-EBV IHCC							
1	57	F	8.5	II	–	–	18.9 months (DOD)
2	68	F	3.8	II	+	–	16.3 months (DOD)
3	54	M	6.7	IVB	+	–	32.9 months (AWD)
4	52	M	5	II	–	–	3.6 months (LTFU)
5	70	M	7.5	II	+	–	28.4 months (AWD)
EBV-LELCC							
1	41	F	1.9	I	+	–	73.3 months (AWOD)
2	53	M	2.7	I	–	–	58.9 months (AWOD)
3	68	F	4.2	IVA	–	–	18.9 months (AWD)
4	70	F	4.5	I	–	–	14.1 months (LTFU)
5	75	M	5.2	III	–	–	66.8 months (AWD)
AWOD, Alive without disease; AWD, alive with disease; DOD, died of disease; LTFU, lost to follow-up; EBV, Epstein-Barr virus; IHCC, intrahepatic cholangiocarcinoma; LELCC, lymphoepithelioma-like cholangiocarcinoma.							
[#] According to American Joint Committee on Cancer 7th edition of IHCC.							

Genetic alteration in EBV-LELCC

Tumor tissue was microdissected according to the results of H&E staining (Fig. 1a) and analyzed using IACCP. The results showed many genetic mutations with an allele frequency (AF) of more than 50%, as shown in Supplementary Tables S1 and S2. Due to the diffuse distribution of TILs over the peritumor area, it was difficult to clearly separate glandular tumor cells from TILs. Sequencing data of germline mutations from PBMCs, that were available for two EBV-LELCC cases, were applied to filter out putative germline mutations. After filtration, mutations with AF of less than or around 10% were considered as somatic. The summary of somatic nonsynonymous mutations in five EBV-LELCC cases is shown in Table 2. Among these mutations, three were identified with COSMIC: CD19 p.R163C (COSM435059), MUC6 p.P1977L (COSM5968299), and PTEN p.Q17* (COSM5153). There were no repetitive mutations found in all EBV-LELCC samples.

Table 2
Somatic nonsynonymous mutations in 5 patients with EBV-LELCC.

Gene Symbol	Amino acid change	Chromosome	Exon	Allele frequency	Total depth
BARD1	p.I143V	2	4	8.10%	371
CD19	p.R163C	16	3	5.60%	787
CD79B	p.D202A	17	6	10.10%	1839
EPHA5	p.H664R	4	11	5.80%	738
KDM5A	p.T30A	12	1	10.20%	979
MUC16	p.R7668T	19	3	7.60%	1089
MUC6	p.P1977L	11	31	6.90%	1949
PTEN	p.Q17	10	1	8.80%	1650
RECQL4	p.V846I	8	16	6.40%	518
TET1	p.R1551G	10	7	7.20%	1397
TNFAIP3	p.A175D	6	4	5.80%	659

Signaling pathways enriched in EBV-LELCC

Five samples each of non-EBV IHCC and EBV-LELCC were extracted for PIPP. As shown in Fig. 1b, 137 differentially expressed genes (DEGs) were identified, including 112 upregulated genes and 25 downregulated genes, in EBV-LELCC compared to non-EBV IHCC. The expression levels of T cell markers, including CD3D, CD3E, and CD8A, and adaptive immune resistance-related genes, such as PDCD1 (PD-1) and CD274 (PD-L1), were enhanced in EBV-LELCC. To understand the major pathways involved in EBV-LELCC, we utilized GO, KEGG, Hallmark, and Reactome databases for enrichment analysis of DEGs. The histograms of the top 40 significantly enriched terms are shown in Fig. 1c, in which the pathway adaptive immune response/system was present in both GO and Reactome databases. Furthermore, GSEA of PIPP data revealed that the genes involved in primary immunodeficiency, adaptive immune response, and adaptive immune system were highly positively enriched in EBV-LELCC (Fig. 1d).

Differences in immune cell types between non-EBV IHCC and EBV-LELCC

We further investigated the major components of innate or adaptive immunity in EBV-infected immune responses between non-EBV IHCC and EBV-LELCC by scoring the corresponding cell type-specific genes based on PIPP. For innate immune response, the scores of mast cells ($P=0.8245$, data not shown), neutrophils ($P=0.9745$, data not shown), dendritic cells (DC), natural killer (NK) cells, NK CD56_{dim} cells, macrophages, M1 macrophages, and M2 macrophages were determined. Among these, only NK CD56_{dim}

cell ($P=0.0181$) and M2 macrophage ($P=0.0304$) scores were found to be higher in EBV-LELCC than in non-EBV IHCC (Supplementary Fig. S1a). In contrast, most immune cell populations involved in adaptive immune responses were generally scored, and were significantly higher in EBV-LELCC than in non-EBV IHCC; such immune cell populations are CD45 cells ($P=0.0416$), B cells ($P=0.0082$), T cells ($P=0.0116$), T helper type 1 (Th1) cells ($P=0.0050$), cytotoxic cells ($P=0.0151$), CD8 T cells ($P=0.0412$), and exhausted CD8 T cells ($P=0.0144$) (Supplementary Fig. S1b).

Expression of PD-L1 and immune cell markers in EBV-LELCC

Based on PIPP data, EBV-LELCC was associated with significantly larger number of immune components of the tumor microenvironment that were related to adaptive immune resistance than that of non-EBV IHCC. We performed IHC or IF staining of PD-L1, tumor cell marker cytokeratin-7 (CK7), and immune cell markers, including CD20, CD56, CD68, CD3, CD4, and CD8 in continuous slides to verify the significantly increased proportion of immune cells in EBV-LELCC and examine which cell types contribute to the development of an immunosuppressive microenvironment. Figures 2a and 2b showed that CD20⁺ B cells, CD56⁺ NK cells, CD68⁺ macrophages, and CD3⁺, CD4⁺, and CD8⁺ T cells migrated to EBV-LELCC. The representative IHC and IF images revealed that PD-L1 was highly expressed in T cells and macrophages compared to tumor cells or the above-mentioned immune cells (Fig. 2a, b, c).

Association between EBV status and the expression of PD-1 and PD-L1

PD-L1 IHC-assessed scores by two independent pathologists showed high consistency ($r=0.8358$, $P=0.0026$; Fig. 3a). Consistent with Figure 2, the distribution of PD-L1 expression by CPS and TPS results showed high CPS but low TPS in all five cases with EBV-LELCC (Fig. 3b and 3c). Indeed, PD-L1 expression is mainly positive in immune cells. Furthermore, a strong correlation was found between PD-L1 expression at the mRNA and protein levels ($r=0.9439$, $P=0.0158$; Fig. 3d). CD274 mRNA expression levels were strongly correlated with PDCD1 mRNA levels in both non-EBV IHCC and EBV-LELCC ($r=0.8205$, $P=0.0036$; Fig. 3e). PDCD1 and CD274 mRNA levels were significantly increased in EBV-LELCC compared to non-EBV IHCC (Fig. 3f), indicating that EBV infection is associated with the expression of PD-1 and PD-L1.

Major components of TIME and clinical outcome in EBV-LELCC

In EBV-LELCC, since the PD-L1 expression was highest in immune cells; abundant in Th1 cells, NK CD56_{dim} cells, M2 macrophages, and CD8 T cells; and exhausted in infiltrated CD8 T cells, we became interested in finding out whether dynamic interactions between these immune cells and cancer cells could evolve tumor progression and immune evasion. The major cellular components of immunity in the two EBV-LELCC cases (E+3 and E+5) showed higher scores for Th1 cells, NK CD56_{dim} cells, and M1 macrophages. However, lower scores for M2 macrophages and exhausted CD8 T cells were observed in

patient E+5 than in patient E+3 (Fig. 4a). The mRNA levels of T cell activation signatures including IL-1B, IL-2, IL-12A, IL-12B, TNF, and IFNG [12] were increased in patient E+5 (Fig. 4b). As compared to patient E+3, patient E+5 had a longer DFS time (29.9 vs. 6.2 months, Fig. 4c) and slow growth rate of aortocaval lymph node metastasis after 2 years follow-up of abdominal computed tomography (CT) scan (2.5 to 2.7 cm, data not shown). Patient E+3 showed progressive disease with multiple mediastinal lymphadenopathies and bilateral pleural effusion after 6 months by CT scan (the largest nodes were 5 and 2.5 cm in size; Fig. 4d and 4e, *left panel*).

Response of EBV-LELCC to anti-PD-1 monoclonal antibody treatment

Patient E+3 was a 68-year-old woman who underwent operation with a diagnosis of stage IVA EBV-LELCC. Due to tumor recurrence, she started receiving chemotherapy with gemcitabine plus cisplatin. However, multiple enlarged mediastinal lymph nodes with bilateral pleural effusion developed later (Fig. 4d and 4e, *left panel*). We changed systemic therapy to nivolumab plus modified gemcitabine and TS-1 (NGS) every 2 weeks [13]. After NGS treatment for 3 months, the multiple metastatic lymphadenopathies obviously decreased bilateral pleural effusion improved which lasted for almost one year (Fig. 4d and 4e, *right panel*). Patient E+5 was a 75-year-old man with a pathological stage III EBV-LELCC received chemotherapy after recurrence. Due to enlarged lymphadenopathy in the aortocaval space (Fig. 4f, *left panel*), he received four cycles of nivolumab monotherapy. Three months later, the post-treatment abdominal CT scan revealed decreased size of the lymphadenopathy with hypodense areas caused by necrosis in the para-aortic and retrocaval regions (4.9 to 3.8 cm and 3.3 to 2.8 cm, respectively) (Fig. 4f, *right panel*). The patient had stable disease under nivolumab treatment for an extended period of 3- or 4-weeks due to a financial issue.

Discussion

The pathological diagnosis of LELCC depends on its morphologic features, including abundant TILs and tumor-associated stroma, but without obvious desmoplastic reaction throughout the tumor [5]. Patients with EBV-LELCC had a significantly longer OS compared to non-EBV IHCC, indicating the unique genetic background and TIME in EBV-LELCC [5]. In this study, we compared EBV-LELCC with non-EBV IHCC in terms of TIME and evaluated the correlation between TIME and prognosis in EBV-LELCC. Higher PD-L1 expression in immune cells, mostly T cells and macrophages, and more activated adaptive immune responses were identified in EBV-LELCC than in non-EBV IHCC. The prognosis of EBV-LELCC appeared to be correlated with the composition and proportion of TIME, in which higher scores of Th1 cells, NK CD56_{dim} cells, and M1 macrophages and lower scores of M2 macrophages and exhausted CD8 T cells were indicators of a relatively hot tumor. Patients with EBV-LELCC experienced tumor shrinkage after treatment with anti-PD-1 monoclonal antibody. Thus, we can consider promoting immunotherapy for the treatment of EBV-LELCC as monotherapy or in combination with chemotherapy.

Approximately 12% of all cancers are associated with viral infections worldwide [14]. Several viruses have been reported to be involved in the development of malignancies, including EBV, human papilloma virus (HPV), and hepatitis B/C viruses (HBV/HCV) [15]. In non-EBV IHCC, *TP53*, *KRAS*, *IDH1*, *BAP1*, *ARID1A*, and *FGFR2* are the most frequently mutated genes [16, 17]. In our study, none of the above genetic mutations were identified in EBV-LELCC cases. In another study, whole exome sequencing revealed that there were no reported well-known mutated genes in EBV-IHCC but non-silent mutations of *MUC4*, *DNAH1*, *GLI2*, *LIPE*, *MYH7*, *RP11-766F14.2*, and *WDR36* were identified, which did not overlap with those of our study [9]. Virus-associated cancers, such as HPV-associated head and neck cancer and EBV-GC, usually show less genetic alternations with a high degree of homogeneity and hypermethylation compared to those without virus infection [8, 18]. Chan et al showed that gene hypermethylation was more frequent in EBV-LELCC than non-EBV IHCC (85.7% vs. 9.1%, $P < 0.003$) [19]. More frequent *pTERT* and *TP53* mutations were found in EBV-negative LELCC than in EBV-LELCC, supporting that EBV-negative LELCC was more likely to be hepatocellular carcinoma or combined hepatocholangiocarcinoma genetically [20, 21]. Due to different etiologies and pathogenesis, EBV-LELCC harbored a different mutational profiling compared to non-EBV IHCC and EBV-negative LELCC.

There are some clinical trials applying anti-PD-1 or anti-PD-L1 monoclonal antibodies for the treatment of BTC after failure to previous chemotherapy, with 5.8-17% of objective response rate [22]. Effective immune biomarkers for patient selection are important in the era of precision medicine. Solid cancers with deficient mismatch repair/microsatellite instability-high and tumor mutation burden-high were tumor-agnostic markers for efficacy of the immune checkpoint inhibitors [23]. In GC, PD-L1 expression and EBV positivity have also been identified as potential biomarkers for the benefit of immunotherapy [24]. In EBV-GC, frequent amplification of *CD274* and higher immune scores were noted compared to non-EBV GC [8]. There was no *CD274* amplification in EBV-LELCC samples by IACCP analysis in our study, and only 5% samples (1 of 20 EBV-IHCCs) were positive as evidenced by FISH in another study [9], suggesting the different mechanisms of PD-L1 overexpression in virus-associated malignancies. Some reports have shown that PD-1 expression is upregulated in T cells during chronic infections and that PD-1 expression is related to T cell exhaustion [25]. Our results revealed significantly high expression of PD-1 and PD-L1 in EBV-LELCC. There was PD-L1 expression on both tumor cells and TILs with higher CPS than TPS, indicating increased expression in TILs, especially in T cells and macrophages.

TIME was significantly related to EBV infection in GC and IHCC [6, 8]. EBV-GC was considered as a hot tumor due to the overexpression of multiple cytokines, chemokines, and HLA genes, which is likely to have inherently strong immune reactions [26]. In EBV-IHCC, a subpopulation of tumor microenvironment types (TMITs), TMIT I, defined by PDL1-tumor⁺/CD8^{high} was more common in EBV-IHCC than in non-EBV IHCC (90.0% vs. 12.4%), which correlated with the best OS compared to TMIT III (PDL1-tumor⁺/CD8^{low}) [9]. Here, we found the activation of adaptive immune response in TIME of EBV-LELCC with enriched PD-1 and PD-L1 expression and abundant Th1 cells, NK CD56_{dim} cells, M2 macrophages, CD8 T cells, and exhausted CD8 T cells infiltration, indicating that EBV-LELCC reflects adaptive immune resistance and may be more sensitive to immunotherapy. In addition, the composition of TIME was associated with DFS

in the two EBV-LELCC cases. The patient E+5 with longer DFS higher scores for Th1 cells, NK CD56_{dim} cells, and M1 macrophages, but lower scores for M2 macrophages and exhausted CD8 T cells than patient E+3, suggesting that the components of TIME may predict clinical course and tumor behavior in EBV-LELCC and can potentially be exploited to enhance the efficacy of immunotherapy.

Because EBV-LELCC is a rare subtype of IHCC, only five samples were enrolled in this study. Further studies with larger sample sizes are needed to validate our results. Furthermore, advanced whole-exon sequencing or whole-genome sequencing is needed to elucidate the pathogenic mechanisms of EBV in carcinogenesis.

Conclusion

EBV-LELCC is a distinct variant of IHCC with dense infiltration of immune cells and different genetic backgrounds and TIMEs. The characteristics of TIME are correlated with clinical outcomes including PD-L1 overexpression in T cells and macrophages, the composition of immune cells, and the degree of T cell activation signatures. EBV-LELCC is a good candidate for the clinical benefit of immunotherapy. Further basket clinical trials of immunotherapy with TIME analysis would be a promising approach to improve the therapeutic outcomes of virus-associated malignancies.

Abbreviations

AF, allele frequency; BTC, biliary tract cancer; CK7, cytokeratin 7; CPS, combined positive score; CT, computed tomography; DC, dendritic cells; DEGs, differentially expressed genes; DFS, disease-free survival; EBV, Epstein-Barr virus; EMRs, electronic medical records; FFPE, formalin-fixed paraffin-embedded; GC, gastric cancer; GO, Gene Ontology; GS, gemcitabine and S-1; GSEA, gene set enrichment analysis; H&E, Hematoxylin and Eosin; IACCP, Ion AmpliSeq Comprehensive Cancer Panel; ICB, immune checkpoint blockade; IHC, immunohistochemistry; IHCC, intrahepatic cholangiocarcinoma; IF, immunofluorescence; INDELs, short insertions/deletions; KEGG, Kyoto Encyclopedia of Genes and Genomes; LELCC, lymphoepithelioma-like cholangiocarcinoma; MAF, minor allele frequency; NK, natural killer; OS, overall survival; PBMCs, peripheral blood mononuclear cells; PDCD1, programmed cell death protein 1; PD-L1, programmed death-ligand 1; PIPP, PanCancer Immune Profiling Panel; qPCR, quantitative real time polymerase chain reaction; SNP, single nucleotide variants; Th1, T helper type 1; Th2, T helper type 2; TIL, tumor-infiltrating lymphocytes; TIME, tumor immune microenvironment; TPS, tumor positive score; VAF, variant allele frequency.

Declarations

Data Availability

The dataset used and/or analysed during the current study are available from the corresponding authors on reasonable requests.

Animal Research (Ethics)

Not applicable

Consent to Participate (Ethics)

This study was approved by the Human Research Ethics Committees of National Cheng-Kung University Hospital (IRB number: B-ER-106-224) and Taipei Veterans General Hospital (IRB number: 2015-03-005BC).

Consent to Publish (Ethics)

All authors had already reviewed and agreed the concept of this manuscript. NJ Chiang, MH Chen and YS Shan have full access to all data and final responsibility for submission and publication of this work.

Plant Reproducibility

Not applicable

Clinical Trials Registration

Not applicable

Author Contributions

NJC, YCH, and MHC designed and drafted the study. NJC, YCH, KTT, HWT, YFH, and MHC analyzed and interpreted the data. NJC, YCH, and KTT used software to analyze genomic and sequencing data. YJL, MHC, LTC, and YSS took care of patients. NJC, YCH, and YSS drafted the manuscript. All authors read and approved the final manuscript.

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Conflict of Interest

YCH created and edited the graphical abstract using BioRender platform (<https://biorender.com/>). NJC received honorarium from AstraZeneca, Bayer, BMS/ONO, MSD, TTY Biopharm. LTC received honorariums from Taiho, TTY Biopharm and Eli Lilly. YSS received honorarium from AstraZeneca, Ipsen, Eli Lilly, Novartis, TTY Biopharm.

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Supplementary Figure

Supplementary Figure 1 is not available with this version.

Figures

Figure 1

Gene-expression signatures and enriched pathways in EBV-LELCC. a Representative images of H&E staining (*left panel*) and EBER-ISH (*right panel*) in two EBV-LELCC cases (E+4 and E+5). Enlarged images on the *middle panel* are shown for the areas outlined by black squares. Original magnification, 200x; scale bars, 100 μ m. EBV-LELCC with positive EBER nuclear staining before microdissection highlighted by blue dotted lines. Dense lymphocytic cells diffusely infiltrate over tumor with lymphoepithelioma-like carcinoma component. **b** Hierarchical clustering heatmap of 137 genes detected as DEGs significantly expressed in non-EBV IHCC (shown as E-) and EBV-LELCC (shown as E+) with false discovery rate - adjusted P value <0.05. Each row represents a single gene, and each column represents a tumor sample. The gene clustering tree is shown on the left. Expression values are represented as colors, where red indicates upregulation and blue downregulation of genes. **c** Histograms of GO, KEGG, Hallmark and Reactome terms are annotated for immune-related pathways. The length of the bars represents the degree of P values obtained from the analysis of each database. The top 40 significantly enriched terms are shown. **d** GSEA enrichment chart of primary immunodeficiency, adaptive immune response, and adaptive immune system signature genes in patients with EBV-LELCC compared with non-EBV IHCC using the KEGG (*left panel*), GO (*middle panel*), and Reactome (*right panel*) pathway datasets. EBV, Epstein-Barr virus; IHCC, intrahepatic cholangiocarcinoma; LELCC, lymphoepithelioma-like cholangiocarcinoma; EBER, Epstein-Barr encoding region; ISH, in situ hybridization; DEGs, differentially expressed genes; GSEA, Gene set enrichment analysis.

Figure 2

Detection of PD-L1 in cancer cells and immune cells from patients with EBV-LELCC. a, b H&E staining and in situ IHC staining for PD-L1, CK7, CD20, CD56, CD68, CD3, CD4, and CD8 in two patients with EBV-LELCC (E+4 and E+5). CK7 was used for identification of cancer cells; CD20 for B cells; CD56 for NK cells; CD68 for macrophages; CD3, CD4, and CD8 for T cells. Original magnification, 200x; scale bars, 100 μ m. **c** IF staining for CD3 or CD68 (green), PD-L1 (red), and nuclear (4',6-diamidino-2-phenylindole, DAPI; blue) staining in serial sections from patient E+4. Expression of PD-L1 (red) and co-localization with CD3 (green, *middle panel*) or CD68 (green, *right panel*) and distribution is shown by overlapping signal (yellow). The white dotted lines indicate the PD-L1 high-expression regions. Original magnification, 400x; scale bars, 50 μ m. EBV, Epstein-Barr virus; LELCC, lymphoepithelioma-like cholangiocarcinoma; PD-L1, programmed death-ligand 1; CK7, cytokeratin-7; NK cells, natural killer cells; H&E, hematoxylin and eosin; IHC, immunohistochemistry; IF, immunofluorescence.

Figure 3

Correlations of EBV infection status with PD-L1 and PD-1 expression. **a** The interpretation of PD-L1 IHC expression levels was evaluated by two independent pathologists with high concordance by Pearson's correlation analysis (r). **b** Distribution of PD-L1 expression is scored by the definition of TPS and CPS in tissue sections from EBV-LELCC patients. **c** The CPS of PD-L1 expression is significantly higher than TPS, with P value <0.01 . **d** Concordant PD-L1 staining score and CD274 mRNA level in EBV-LELCC tissues (Pearson's correlation coefficient, r). **e** Pearson correlation coefficient (r) between PDCD1 and CD274 mRNA expression levels in non-EBV IHCC and EBV-LELCC tissues. **f** PDCD1 and CD274 mRNA expression levels were measured and expressed by qPCR. *, $P<0.05$ compared with EBV- group, as determined using two-way ANOVA. All values are the mean $\pm$ s.e.m. TPS, tumor proportion score; CPS, combined positive score; EBV-, non-EBV IHCC; EBV+, EBV-LELCC; EBV, Epstein-Barr virus; LELCC, lymphoepithelioma-like cholangiocarcinoma; PD-L1, programmed death-ligand 1; PDCD1, Programmed cell death protein 1.

Figure 4

The major immunoregulatory cells, prognosis, and efficacy of nivolumab in EBV-LELCC patients. **a** The scores of immunoregulatory cell types including Th1 cells, NK CD56_{dim} cells, M1 macrophages, M2 macrophages, and exhausted CD8 T cells in two EBV-LELCC patients (E+3 and E+5). **b** The mRNA expression levels of IL1B, IL2, IL12A, IL12B, TNF, IFNG were analyzed in patient E+3 and E+5. **c** The bar chart for patient E+3 and E+5 indicating the disease-free survival time. **d, e** CT scans on the EBV-LELCC

patient E+3 showing the different stages of metastatic mediastinal lesions (blue arrows) in the chest over time. The images of mediastinal lymph nodes (**d**) and pleural seeding (**e**) in the patient E+3 with pre- (*left panel*) and post-treatment (*right panel*) of nivolumab plus modified GS, respectively. **f** Abdominal CT scan of metastatic lymphadenopathy in para-aortic and retrocaval regions before (blue arrows, 4.9 and 3.3 cm, respectively) (*left panel*) and after (blue arrows, 3.8 and 2.8 cm, respectively) (*right panel*) the treatment of nivolumab monotherapy in patient E+5. EBV, Epstein-Barr virus; LELCC, lymphoepithelioma-like cholangiocarcinoma; Th1 cells, T helper type 1 cells; NK cells, natural killer cells; CT, computed tomography; nivolumab, anti-PD-1 monoclonal antibody; GS, gemcitabine and S-1.

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