Hepatitis B Virus-Associated Follicular Lymphoma Reveals T-Cell Inflamed Phenotype and Response to Lenalidomide

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Abstract

Background: Follicular lymphoma (FL) is a malignancy of lymphocytes derived from germinal center (GC). Hepatitis B virus (HBV) infection may increase the incidence of FL. Here, we performed an integrated genomic and transcriptomic analysis to identify molecular characteristics and therapeutic targets for HBV-associated FL.

Methods: A total of 253 patients with newly diagnosed FL were enrolled. Whole-genome sequencing (n=13) and whole-exome sequencing (n=87) were performed on tumor samples of 100 patients. Among them, 84 patients were available for transcriptomic sequencing data. Moreover, biological function of immune modulator TNFAIP3 mutation was investigated in vitro using FL cell line SC-1 and in vivo using zebrafish xenograft model.

Results: By characterizing altogether 253 FL patients, we showed that patients with HBV infection (surface antigen positive, HBsAg+) were significantly associated with younger age, more frequent spleen involvement, advanced histological grade, higher proliferation index, and poorer progression-free survival. By whole-genome/exome and targeted sequencing, we observed increased mutations in immune modulators (TNFAIP3, CD70, CXCR4, PIM1, KLF2, FAS, CD58, and CD83), decreased mutations in epigenetic modifiers (KMT2D and CREBBP), and low incidence of BCL2 translocation as the core oncogenic program of HBsAg+ FL. By transcriptomic sequencing, we further showed that HBsAg+ FL was enriched by immune-related pathways, antigen processing and presentation and IFN signatures, as well as inflamed signatures and infiltration of CD8+T and Th1 cells, with malignant lymphocytes transiting to post-GC phenotype. In the co-culture system of FL cell line SC-1 with peripheral blood mononuclear cells mimicking in vivo situation, TNFAIP3 mutations induced overexpression of TIGIT on CD8+T cells and VISTA on Th1 cells, both of which were downregulated by lenalidomide, resulting in sensitivity of TNFAIP3-mutant cells to lenalidomide treatment in co-culture system and in zebrafish xenograft model. Of note, negative prognostic impact of HBV infection on patients treated with rituximab-based immunochemoetherapy could be overcome by rituximab and lenalidomide.

Conclusions: HBV-associated FL may be considered as a specific subtype, based on distinct immune modulator mutations, and CD8+T- and Th1-enriched post-GC phenotype. Moreover, these findings provided new insights into the pathogenetic role of HBV in FL and the potential benefit of immunomodulatory agents in treating HBV-associated FL.

Background

Follicular lymphoma (FL) represents the most common subtype of indolent non-Hodgkin's lymphoma with distinct pathological, cytogenetic, and molecular features [1, 2]. In pathology, FL maintains a stage of differentiation similar to germinal center B (GCB) cells, with positive expression of CD10, BCL6, and BCL2 in most patients and negative expression of MUM1 [1, 2]. Based on the centrocytes/centroblasts in GC, FL is divided into FL grade 1 (FLI), FL grade 2 (FLII), FL grade 3A (FLIIIA), and FL grade 3B (FLIIIB) [2].
In terms of cytogenetic and molecular aberrations, up to 90% of FL patients harbor the t(14;18) (q32;q21)/BCL2-IGH translocation, as well as mutations in epigenetic modifiers, particularly KMT2D and CREBBP [1,2]. In addition to malignant cells themselves, FL is composed of various immune cells, including CD8+ T cells, CD4+ T helper cells, CD4+ T regulatory cells, and dendritic cells [3]. The tumor microenvironment plays crucial roles in FL development and clinical outcome [3]. For example, CD8+ T cells can be activated by the engagement of T cell receptor (TCR) with complexes formed between major histocompatibility complex (MHC) I modules and antigenic peptides on FL B cells [4]. CD4+ T helper cells are able to engage TCR in cellular interaction with FL B cells via MHC II modules [5].

Growing evidence has suggested virus infection may contribute to the pathogenesis of malignant lymphoma, such as Epstein Barr virus, human T-cell lymphotropic virus, human herpes virus-8, human immunodeficiency virus, and hepatitis C virus [6,7]. As an oncogenic virus of liver cancer, hepatitis B virus (HBV) is also recognized as a potential pathogenic factor in B cell lymphoma, based on the epidemiological evidence that individuals with HBV infection have a high risk of developing diffuse large B cell lymphoma (DLBCL) and FL [8-10]. Gene expression signatures regulated by BCL6, FOX01, and ZFP36L1 was enriched in HBV-associated DLBCL [11]. However, molecular characteristics underlying the pathogenetic role of HBV infection have never been reported in FL. Recently, diversified novel targeted agents have proven effective in treating FL, including targeting hallmark BCL2 overexpression (BCL2 inhibitor), epigenetic dysregulation (e.g., EZH2 inhibitor), and tumor microenvironment alterations (e.g., lenalidomide, anti-PD-1 antibody) [12]. Of note, rituximab plus lenalidomide (R2 regimen) show promising activity and long-term benefit as first-line treatment in FL [13]. Targeted agents warrant further investigation for mechanism-based treatment in HBV-associated FL.

In the present study, we performed a first integrated genomic and transcriptomic analysis to elucidate the pathogenetic role of HBV infection in FL, and to investigate potential therapeutic approaches targeting genetic and microenvironmental vulnerabilities of HBV-associated FL.

Methods

Patients

From January 2011 to October 2020, a total of 253 patients with newly diagnosed FL were enrolled. Histological diagnoses were reviewed by two independent pathologists (L.D. and H.-M.X.) based on the World Health Organization classification [14]. Seventy-eight patients were from a prospective clinical trial, R2 in the Treatment of Follicular Lymphoma (NCT03715309), and 175 patients were from a retrospective cohort receiving rituximab-based immunochemotherapy (R-CHOP, rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisone, n=165; R-CVP, rituximab, cyclophosphamide, vincristine, and prednisone, n=7; R-FCM, rituximab, fludarabine, cyclophosphamide, and mitoxantrone, n=2; BR, rituximab and bendamustine, n=1). Detection of HBV surface-antigen (HBsAg), HBV e-antigen (HBeAg), antibodies against HBsAg (HBsAb), antibodies against HBeAg (HBeAb), and antibodies against HBV core-antigen (HBcAb) were performed as routine blood tests. The quantitation of HBV DNA was performed in HBsAg+
patients. All patients were classified into HBsAg+ (concomitant infection, n=43) and HBsAg- (n=210) groups, and HBsAg- FL patients were subsequently divided into HBsAg-/HBcAb+ (occult infection) and HBsAg-/HBcAb- (uninfected), as previously reported [11, 15]. The study was approved by the Ruijin Hospital Ethics Committee with written informed consent obtained in accordance with the Declaration of Helsinki.

**Genomic and transcriptomic analysis**

DNA was extracted from tumor samples and peripheral blood samples using a QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) or GeneRead DNA FFPE Tissue Kit (Qiagen, Hilden, Germany). Total RNA was extracted using RNeasy Mini Kit (Qiagen, Hilden, Germany). Whole-genome sequencing (WGS) were performed on 13 tumor samples, whole-exome sequencing (WES) were performed on 87 tumor samples, and targeted sequencing were performed on 9 tumor samples. Among them, WES (n=29) and WGS (n=12) were performed on 41 matched peripheral blood samples randomly selected. Transcriptomic sequencing was performed on 84 qualified tissues. Sanger sequencing were further used to confirm somatic mutations not observed in matched peripheral blood mononuclear cells (PBMCs). None of the mutations were detected in matched PBMCs from corresponding patients. Detailed sequencing protocols and bioinformatic principles were shown in the supplementary appendix. The detailed methods were shown in Additional file 1: Supplementary methods.

**Immunohistochemistry**

Immunohistochemistry was performed on 4 mm paraffin sections using antibodies against CD10 (56C6, DAKO), BCL2 (124, DAKO), BCL6 (PG-B6p, DAKO), MUM1 (MUM1p, DAKO), Ki67 (MIB-1, DAKO), and HBsAg (Abcam), as previously reported [16]. Protein expression levels were scored based on percentage of stained cells, with CD10, BCL6 and MUM1 staining ≥ 30%, and BCL2 staining ≥ 50% as positive. Ki67 was assessed semi-quantitatively (increments of 5%, i.e., 5%, 10%, 15%) [16].

**Western blot**

Proteins were extracted using NE-PER Nuclear and Cytoplasmic Extraction Reagents (78835, Thermo Fisher Scientific). The immunocomplexes were visualized using a NF-κB Family Antibody Sampler Kit II (Cell Signaling Technologies (CST), Danvers, MA, USA). Each primary antibody was validated for the relevant species and applications, as shown on the manufacturers’ websites, including p65 (8242, CST), Relb (10544, CST), c-Rel (12707, CST), p105/p50 (13586, CST), and p100/P52 (37359, CST). GAPDH (2118, CST), and Lamin B1 (13435, CST) were used to ensure equivalent loading of protein. Horseradish peroxidase-conjugated secondary IgG antibodies against rabbits were from CST.

**Quantitative Real-Time PCR (RT-PCR)**

Total mRNA was extracted using RNeasy Mini Kit (Qiagen, Hilden, Germany). Complementary DNA was synthesized using PrimeScript RT Reagent Kit with gDNA Eraser (TaKaRa, Dalian, China). RT-PCR was
performed by SYBR Premix Ex Taq™ II (TaKaRa) and ABI ViiA 7 (Applied Biosystems) with primers against *TNFAIP3* and *GAPDH* was used as an endogenous control.

**Fluorescence in situ hybridization (FISH)**

FISH was performed on 3 mm paraffin tissue sections using *BCL2* fusion probes (F01008-01, Beijing Jinpujia), as previously reported [17]. Signaling was counted in 200 cells and considered positive if more than 10% of the tumor cells exhibited a break apart signal [16].

**Cell lines**

FL cell line SC-1, available from DSMZ Cell Lines Bank ([https://www.dsmz.de/dsmz](https://www.dsmz.de/dsmz)), was cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) in a humidified atmosphere of 95% air and 5% CO$_2$ at 37°C. PBMCs were isolated from peripheral blood with ficoll using density gradient centrifugation [18].

**Cell transfection**

Cells were transfected with viral particles containing purified plasmids GV367-EGFR-puromycin-TNFAIP3-WT, GV367-EGFR-puromycin-TNFAIP3-L147fs, GV367-EGFR-puromycin-TNFAIP3-R706X, as well as GV367-EGFR-puromycin-vector (MOI=50) using Lipofectamine 3000 transfection reagents (Invitrogen) according to the manufacturer’s instruction. The stably transfected clones were selected by GFP using BD FacsAria.

**Cell viability**

Cells (2×10$^5$/ml) were seeded in 96-well plates and incubated with indicated concentration of reagents. Cell growth was assessed by CCK8 (1:10, Dojindo, Kumamoto, Japan) and the absorbance was measured at 450 nm by spectrophotometry. The percentage of cell growth inhibition was calculated as transfected cells divided by treated or untreated cells.

**Flow cytometry**

Co-cultured SC-1 cells and PBMCs were stained with anti-CD19 (555415, BD Pharmingen), anti-CD3 (612940, BD Pharmingen), anti-CD4 (624298, BD Pharmingen), anti-CD8 (563919, BD Pharmingen), anti-T-bet (624295, BD Pharmingen), anti-GATA3 (560405, BD Pharmingen), anti-TIGIT (747846, BD Pharmingen), and anti-VISTA (624296, BD Pharmingen) antibodies to assess the proliferation and immune receptor expression of tumor cells and immune cells. The cells were collected by BD FACSymphony A5.

**Xenograft models in zebrafish**

Dil-labeled cells were injected into the perivitelline space (PVS) of anesthetized 48-hpf wild type Tuebingen zebrafish larvae and treatment started at 24h post injection as previously reported [19]. Censor
of sensitivity was death event of zebrafish in the following 5 days. The concentration of drugs was determined based on patient plasma concentration as reported [20]. All animal experiments were approved by the Animal Care and Use Committee of Shanghai Jiao Tong University. All animal experiment procedures followed the rules of the Committee on Animal Care of Shanghai, China and the ARRIVE guidelines [21].

**Statistical analysis**

Baseline characteristics were analyzed using Fisher’s exact test. The unpaired t tests were applied to compare two normally distributed groups and Mann-Whitney U tests were applied to compare nonparametric continuous variables. Progression-free survival (PFS) was calculated from the date of diagnosis to the date when the disease progression was recognized or the date of last follow-up. Overall survival (OS) was measured from the date of diagnosis to the date of death or the date of last follow-up. Survival analyses were estimated using the Kaplan-Meier method and compared by log-rank test. Statistical significance was defined as two-sided $p$ value <0.050. All statistical analyses were carried out using Statistical Package for the Social Sciences (SPSS, 26.0) or GraphPad Prism 7 software.

**Results**

**Overall clinicopathologic and genetic features of FL**

Clinicopathologic characteristics of 253 patients with FL were summarized in Table 1. To further explore the genetic features of FL, we performed DNA sequencing on 109 patients (WGS, n = 13; WES, n = 87; and targeted sequencing, n=9). Among them, a median of 59 (37-120) and 58 (3-131) somatic mutations in the coding regions per tumor tissue were identified by WES and WGS, respectively (Additional file 2: Figure S1A-C). A preference for missense mutation (Figure 1A) and G/C>A/T alteration (Figure 1B) were observed, including somatic single nucleotide variations (SNVs) and small insertions/deletions (INDELs), in analogous to the somatic SNV spectrum in other cancers [22]. Fifty-six candidate genes with predicted functional alterations and related to FL pathogenesis were analyzed. As shown in Figure 1C, KMT2D and CREBBP were the most frequently mutated genes, followed by BCL2, TNFRSF14, STAT6, EZH2, CARD11, ARID1A, and EP300. Seventy-one patients were positive for BCL2 translocation. We noted significantly decreased BCL2 translocation (Additional file 2: Figure S1D, $p < 0.001$) and mutations in KMT2D (Additional file 2: Figure S1E, $p = 0.010$) and CREBBP ($p = 0.042$) when compared with the previous literature in Western population [23].

**Clinicopathologic and genetic features of HBV-associated FL**

Among 253 patients, 17.0% patients (43/253) were HBsAg+ (Table 1). HBV DNA was quantified in all HBsAg+ FL (Additional file 3: Table S1) and below $10^3$ IU/ml before rituximab-containing treatment. As shown in Table 1, HBsAg+ FL presented significantly younger age ($p = 0.044$), more frequent spleen involvement ($p = 0.006$), more histological grade FLIIIA ($p < 0.001$), and higher proliferation index (Ki67) ($p < 0.001$). As for HBsAg- subgroups, no significant difference was observed between HBsAg-/HBcAb-
and HBsAg-/HBcAb+ patients, except that HBsAg-/HBcAb+ patients were older than HBsAg-/HBcAb- patients (Additional file 3: Table S2), probably due to HBV vaccination for newborns since 1992 [24].

Genetic aberrations were compared in 33 HBsAg+ FL and 76 HBsAg- FL. Totally, four clusters of genetic aberrations were defined according to gene functions (Figure 2A). HBsAg+ FL patients were significantly associated with increased mutations in immune modulators including TNFAIP3, CD70, CXCR4, PIM1, KLF2, FAS, CD58, and CD83, and decreased mutations in epigenetic modifiers including KMT2D and CREBBP. In terms of functional clusters (Figure 2B), higher mutations in immune modulators (p < 0.001), but lower mutations in epigenetic modifiers (p < 0.001) were observed in HBsAg+ FL. As for HBsAg- subgroups, no significant difference was observed between HBsAg-/HBcAb- and HBsAg-/HBcAb+ patients in genetic aberrations (Additional file 3: Table S3) or functional clusters (Additional file 3: Table S4).

Besides, decreased BCL2 translocation (p < 0.001) and BCL2 expression (p = 0.003) were detected in HBsAg+ FL patients (Figure 2C). As for HBsAg- subgroups, no significant differences were observed between HBsAg-/HBcAb+ and HBsAg-/HBcAb- patients (Additional file 3: Table S5). Meanwhile, different from those in hepatocellular carcinoma (e.g., viral gene integration into the liver cell genome) [25]. integration of viral genes into the genome of tumor cells was not detected in HBsAg+ FL using WGS, and HBsAg was not detected by immunohistochemistry method (Additional file 2: Figure S2A-S2C).

**Enriched immune-related pathways in HBV-associated FL**

Using transcriptomic sequencing, gene expression patterns of 17 HBsAg+ FL and 67 HBsAg- FL were compared. Overall, 1514 differentially expressed genes (DEGs) were identified (Additional file 3: Table S6). Enriched Gene Ontology (GO) terms of DEGs revealed that HBsAg+ FL was closely related to immune-related pathways, including T-cell activation, leukocyte differentiation, positive regulation of innate immune response, negative regulation of immune system process, activation of innate immune response, lymphocyte differentiation, antigen processing and presentation, TCR, NIK/NF-κB, positive regulation of leukocyte activation, TNF-mediated, and B-cell activation signaling pathways (Figure 3A). Moreover, ingenuity pathway analysis (IPA) of upstream regulators observed a number of inflammatory and effector cytokine pathways were activated in HBsAg+ FL, including interferon (IFN)-γ, TNF, CD3 complex, IFN-α, NF-κB complex, TCR complex, and B cell receptor (BCR) complex, while PTEN, BCL6, and BACH2 were inhibited (Figure 3B). Gene set enrichment analysis (GSEA) analysis confirmed significantly upregulated TCR signaling pathway (Additional file 2: Figure S3A) and TNF signaling pathway via NF-κB (Additional file 2: Figure S3B) in HBsAg+ FL. Altogether, these findings suggested that HBsAg+ FL exhibited an immune-inflamed phenotype.

**Enriched antigen processing and presentation and IFN signatures in HBV-associated FL**

As reported, immune-inflamed tumors were usually characterized by enhanced antigen processing and presentation, activation of IFN signatures, and infiltration of CD8+ T cells [26, 27]. Interestingly, antigen processing and presentation was indeed enriched in HBsAg+ FL according to GO terms (Figure 3A), and
GSEA confirmed this (Additional file 2: Figure S3C). Since MHC molecules were well-known for their role in antigen presentation [28], we evaluated the association of MHC molecules with HBsAg+ FL. Significantly higher expression of MHC class I molecules were observed in HBsAg+ FL (Figure 3C), including $\text{HLA-A} (p = 0.001)$ and $\text{HLA-C} (p = 0.002)$. As for MHC class II molecules (Figure 3D), significantly higher expression of $\text{HLA-DPB1}$, $\text{HLA-DQA1}$, $\text{HLA-DQA2}$, $\text{HLA-DQB1}$, $\text{HLA-DQB2}$, $\text{HLA-DRB1}$, and $\text{HLA-DRB5}$ was observed in HBsAg+ FL.

As aforementioned in Figure 3B, IFN-γ and IFN-α were activated upstream regulators in HBsAg+ FL. Indeed, expression of IFN signatures were significantly upregulated in HBsAg+ FL (Figure 3E), including 10-gene signature, 28-gene signature, 6-gene signature, and 18-gene signature as reported by Terrill K. McClanahan [29]. The detailed genes of signatures were summarized in Additional file 3: Table S7.

**Enriched infiltration of CD8$^+$T and Th1 cells in HBV-associated FL**

T-cell subsets were distinguished by the xCell method using transcriptomic sequencing data (Figure 3F) [30]. HBsAg+ FL presented significantly increased total CD8$^+$T cells ($p = 0.010$) and increased CD8$^+$T cell subsets, including CD8$^+$naïve T cell ($p < 0.001$), CD8$^+$central memory T cell (CD8$^+$Tcm, $p = 0.003$), and CD8$^+$effector memory T cell (CD8$^+$Tem, $p = 0.003$). As for CD4$^+$T cell, Th1 cells were significantly higher in HBsAg+ FL ($p < 0.001$). Significant upregulation of individual genes related to CD8$^+$naïve T cells, CD8$^+$Tcm, CD8$^+$Tem, and Th1 cells was also found in HBsAg+ FL (Figure 3G). The detailed genes of T-cell subsets were listed in Additional file 3: Table S8 as described by the xCell method [30]. Therefore, we concluded that HBV-associated FL was characterized by T-cell inflamed phenotype of CD8$^+$T and Th1 cells.

**Increased class-switched memory B cell signatures in HBV-associated FL**

Since B cells can be efficiently activated by antigens under the help of T cells [31, 32], B-cell activation signaling pathway was enriched in HBsAg+ FL according to GO terms in Figure 3A, and BCR signaling pathway was upregulated according to GSEA (Additional file 2: Figure S3D). Meanwhile, activated upstream regulators in HBsAg+ FL included BCR complex (Figure 3B). When comparing the difference of B-cell subsets by the xCell [30], we observed decreased naïve B cells ($p < 0.001$) and increased class-switched memory B cells (MBCs) ($p = 0.020$) in HBsAg+ FL (Figure 4A). Indeed, expression of individual genes related to naïve B cells was downregulated, while expression of individual genes related to class-switched MBCs was upregulated in HBsAg+ FL (Figure 4B). The detailed genes of B-cell subsets were listed in Additional file 3: Table S8 as described by the xCell method [30]. The finding of increased class-switched MBCs in HBsAg+ FL suggested increased ongoing immune response and post-GC B cells upon chronic HBV antigen stimulation [33, 34].

**Increased post-GC signatures in HBV-associated FL**

Expression of the major transcription factors involved in GC development were subsequently analyzed (Figure 4C, left panel) [35, 36]. Significantly downregulation of genes involved in early GC initiation, late
GC initiation, and GC maintenance (POU2AF1, MEFC, IRF8, BCL6, BACH2, and EBF1), while upregulation of genes involved in post-GC (IRF4 and PRDM1) were observed in HBsAg+ FL. Indeed, BCL6 and BACH2 were inhibited upstream regulators in HBsAg+ FL as shown in Figure 3B. Expression of key surface markers on GC B and post-GC B cells were also screened (Figure 4C, right panel) [37-39]. In parallel with the expression of transcription factors, lower expression of surface markers on GC B cells (CD10), but higher expression of surface markers on post-GC B cells (CD44) were observed in HBsAg+ FL. Using immunohistochemistry, protein expression of BCL6, MUM1, and CD10 were confirmed (Figure 4D), with significantly decreased expression of BCL6 (p = 0.005) and CD10 (p = 0.002), and increased expression of MUM1 (p < 0.001) in HBsAg+ FL.

Moreover, cell-of-origin (COO) was examined by transcriptomic sequencing data using the method previously reported in DLBCL [40]. Interestingly, HBsAg+ FL exhibited increased activated B cell like (ABC-like) subtype (p < 0.001, Figure 4E), which was reported to derive from post-GC B cells [36]. Detailed unsupervised clustering heatmap was shown in Additional file 2: Figure S4A. These data indicated that HBV-associated FL tumor cells were featured by a post-GC phenotype.

**Increased CD8^+T and Th1 cells in PBMCs co-cultured with TNFAIP3-mutant cells**

To explore the role of immune modulator mutations in HBV-associated FL, the function of TNFAIP3 mutation was investigated. TNFAIP3-wt and TNFAIP3-mutant (L147fs, R706X) plasmids were transfected into the SC-1 cell line, and the transfection efficiency was confirmed by sequencing of RT-PCR products (Additional file 2: Figure S5A). Enhanced cell proliferation was observed in TNFAIP3-mutant cells (Figure 5A). Upregulated transcriptional factors involved in NF-κB signaling pathway were also confirmed in TNFAIP3-mutant cells by western blot (Additional file 2: Figure S5B).

When co-cultured with PBMCs, a significant increase in CD8^+T cells, and higher expression of TIGIT on CD8^+T cells were observed in TNFAIP3-mutant cells (Figure 5B). We also found a significant increase in Th1 cells and higher expression of VISTA on Th1 cells in TNFAIP3-mutant cells (Figure 5C).

**Evidence of lenalidomide sensitivity in TNFAIP3-mutant cells in vitro and in vivo**

TNFAIP3-mutant cells showed increased sensitivity to lenalidomide, with lower half maximal inhibitory concentration [IC_{50}] at 48h and 72h (Figure 5D). When co-cultured with PBMCs, TNFAIP3-mutant cell growth was significantly inhibited by lenalidomide treatment (Figure 5E). Meanwhile, CD8^+T cells of the co-cultured system with TNFAIP3-mutant cells were significantly increased, while the expression of TIGIT on CD8^+T cells were decreased (Figure 5F). Th1 cells of the co-cultured system with TNFAIP3-mutant cells were significantly increased, while the expression of VISTA on Th1 cells were decreased (Figure 5G).

In vivo, zebrafish xenograft models showed decreased survival when injected with TNFAIP3-mutant cells (Figure 5H, upper panel), and prolonged survival upon lenalidomide treatment (Figure 5H, lower panel).

**Clinical evidence of rituximab and lenalidomide sensitivity in HBV-associated FL**
In clinical settings, significantly increased CD8⁺T cells, Th1 cells, as well as expression of TIGIT, and VISTA were observed in the tumor tissues with HBV-associated immune modulator mutations (TNFAIP3/CD70/CXCR4/PIM1/KLF2/FAS/CD58/CD83 mutations, Figure 6A). These findings suggested that lenalidomide may be more effective in HBsAg+ FL. Survival analysis was performed on 253 patients treated with rituximab-based immunochemotherapy or R2 regimen, including 43 HBsAg+ FL and 210 HBsAg- FL patients. The median follow-up time was 18.8 (range 2.0-88.1) months. For rituximab-based immunochemotherapy, HBsAg+ FL presented inferior PFS (p = 0.006, Figure 6B), with the 2-year PFS rate of 82.2%. Of note, for R2 regimen, there was no significant difference in PFS (p = 0.217, Figure 6C) between HBsAg+ FL and HBsAg- FL, with the 2-year PFS rate of 100.0% and 82.5%, respectively. Therefore, rituximab and lenalidomide appeared to abrogate a negative effect of HBV infection on FL outcome upon rituximab-based immunochemotherapy.

Discussion

To our knowledge, this is the first genome-wide analysis on HBV-associated FL. In accordance with the epidemiology data that HBV is related to the pathogenesis of FL [41], we discovered distinct molecular features of HBV-associated FL, including decreased BCL2 translocation, decreased mutations in epigenetic modifiers, such as KMT2D and CREBBP, but increased mutations in immune modulators, such as TNFAIP3, CD70, CXCR4, PIM1, KLF2, FAS, CD58, and CD83. This is in consistence with a previous report on 13 BCL2 translocation-negative FL patients who show decreased mutations in KMT2D, CREBBP, and increased mutations in immune response [42]. Among these mutations in immune modifiers, TNFAIP3 and KLF2 are implicated in negative regulation of inflammation through counteracting NF-κB activation [43, 44]. TNFAIP3 deficiency increases infiltration of CD8⁺T cells in melanoma [44, 45], and knockout of KLF2 enhances T cell activation and immune response [46]. CD70 and CD58 act as co-stimulatory molecules to promote T cell function through binding to CD27 and CD2, respectively [47, 48]. CD83 is an untypical co-stimulatory molecule and amplifies CD8⁺T cells through the engagement of CD83 ligand [49, 50]. FAS contributes to T-cell-mediated immune surveillance via binding of FAS ligand [51]. CXCR4 is known as a chemokine receptor, co-stimulating TCR activation in T cells [52, 53]. PIM1 is also involved in the proliferation and function of CD8⁺T cells [54]. Interestingly, most of the mutations in immune modulators aforementioned have been reported more frequently in T-cell inflamed DLBCL [55, 56]. Indeed, our transcriptomic data further confirmed a T-cell inflamed phenotype in HBV-associated FL, manifested by upregulation of TCR, antigen processing and presentation, TNF, NF-κB, and IFN signatures, as well as increased infiltration of CD8⁺T and Th1 cells [26, 27, 55]. Another prominent feature of the inflamed lymphomas is their ability to facilitate immune escape, particularly due to upregulation of inhibitory immune checkpoint molecules [26, 27, 55]. Consistently, higher expression levels of inhibitory immune checkpoint molecules TIGIT and VISTA were observed in CD8⁺T and Th1 cells, respectively. TIGIT is an inhibitory receptor expressed on T cells, and inhibits T cell function via interacting with CD155 or CD122 [57]. VISTA is constitutively expressed on T cells and myeloid cells, and negatively regulates T cell response [58].
The microenvironment, especially antigenic stimulations and interactions with T cells, play essential roles in determining the development of GC B cells [59]. Using transcriptomic sequencing data, we identified a post-GC phenotype of HBV-associated FL, manifested by downregulation of transcriptional factors driving a “GC B cell transcriptional program”, including \textit{POU2AF1}, \textit{MEF2C}, \textit{IRF8}, \textit{BCL6}, \textit{BACH2}, and \textit{EBF1}, and upregulation of transcriptional factors involved in a “post-GC program”, including \textit{IRF4} and \textit{PRDM1} [36]. As reported, \textit{POU2AF1} and \textit{MEF2C} are both key molecules involved in the early GC initiation, and \textit{IRF8} contributes to induction of \textit{BCL6}, which is essential for the late GC initiation and GC maintenance [35, 36]. \textit{BACH2} and \textit{EBF1} are critical in GC maintenance [35, 36]. \textit{IRF4} and \textit{PRDM1} are two major transcriptional factors initiating the post-GC program upon NF-κB activation [35, 36]. Moreover, pivotal B cell markers exhibited a similar trend to a post-GC phenotype in HBV-associated FL, including decreased expression of \textit{CD10}, which is a GC B cell marker and gradually lost during post-GC [37, 60, 61], and increased expression of \textit{CD44}, which is upregulated during B cell transmitting to post-GC [38]. In fact, it has been reported that the post-GC signature is enriched in \textit{BCL2} translocation-negative FL, who are characterized by decreased CD10 expression and increased MUM1 expression [62, 63]. Based on these findings, we propose a model in which HBV infection induces a T-cell inflamed phenotype of FL manifested by frequent mutations in immune modulators, continuous activation of TCR, antigen processing and presentation, TNF, NF-κB, and IFN signatures, as well as increased CD8⁺T and Th1 cell infiltration. Meanwhile, T-cell inflamed status contributes to a hyper-active status of B cells (post-GC phenotype) and subsequent malignant progression of B cells. Our data suggest an alternative mechanism of FL pathogenesis, independent of \textit{BCL2} translocation and mutations in epigenetics modifiers [1].

Recently, lenalidomide has shown promising efficacy in treating FL [13, 64, 65]. According to previous literature, lenalidomide exerts anti-tumor activity through both direct and indirect manners [65]. The direct activity is mediated by downregulation of NF-κB signatures in an \textit{IRF4}-dependent manner, as reported in ABC-DLBCL [66, 67], in accordance with our finding of increased sensitivity to lenalidomide in \textit{TNFAIP3}-mutant cells. The indirect activity mainly includes stimulating tumor-infiltrating CD8⁺T and T helper cell activity, and targeting inflammatory pathways, such as TNF, NF-κB, IFN, and antigen presentation signatures [56, 65]. Interestingly, infiltration of CD8⁺T and T helper cells, as well as inflammatory pathways are significantly increased in HBV-associated FL. Using \textit{in vitro} co-culture system, we showed the inhibitory effect of lenalidomide on TIGIT and VISTA expression in \textit{TNFAIP3}-mutant cells, both of which may serve as promising therapeutic targets for HBV-associated FL. Further in FL patients, rituximab and lenalidomide appeared to mitigate a negative impact of HBV infection on clinical outcome upon rituximab-based immunochemotherapy. Although larger cohort studies are needed to validate the clinical efficacy of R2 regimen, our results demonstrated the potential effect of lenalidomide against the immune alterations in HBV-associated FL.

\textbf{Conclusions}
HBV-associated FL may be considered as a specific subtype, based on distinct immune modulator mutations, and CD8+T- and Th1-enriched post-GC phenotype. Potent immunomodulatory effect of lenalidomide on HBV-associated FL provide clinical rationale of enhancing anti-tumor immune surveillance in treating virus-associated B-cell lymphoid malignancies.

**Abbreviations**

FL: follicular lymphoma; GC: germinal center; HBV: hepatitis B virus; GCB: germinal center B; FLI: FL grade 1; FLII: FL grade 2; FLIIIA: FL grade 3A; FLIII: FL grade 3B; TCR: T cell receptor; MHC: major histocompatibility complex; DLBCL: diffuse large B cell lymphoma; R2 regimen: rituximab plus lenalidomide; R-CHOP: rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisone; R-CVP: rituximab, cyclophosphamide, vincristine, and prednisone; R-FCM: rituximab, fludarabine, cyclophosphamide, and mitoxantrone; BR: rituximab and bendamustine; HBsAg: HBV surface-antigen; HBeAg: HBV e-antigen; HBsAb: antibodies against HBsAg; HBeAb: antibodies against HBeAg; HBCAb: antibodies against HBV core-antigen; WGS: whole-genome sequencing; WES: whole-exome sequencing; PBMCs: peripheral blood mononuclear cells; FISH: fluorescence in situ hybridization; FBS: fetal bovine serum; PVS: perivitelline space; PFS: progression-free survival; OS: overall survival; SPSS: Statistical Package for the Social Sciences; SNVs: single nucleotide variations; INDELs: insertions/deletions; DEGs: differentially expressed genes; GO: Gene Ontology; IPA: ingenuity pathway analysis; IFN: interferon; BCR: B cell receptor; GSEA: gene set enrichment analysis; CD8+Tcm: CD8+ central memory T cell; CD8+Tem: CD8+ effector memory T cell; MBCs: class-switched memory B cells; COO: cell-of-origin; ABC-like: activated B cell like; RT-PCR: Quantitative Real-Time PCR; IC\textsubscript{50}: half maximal inhibitory concentration.

**Declarations**

**Ethics approval and consent to participate**

The study was approved by the Ruijin Hospital Ethics Committee with written informed consent obtained in accordance with the Declaration of Helsinki. And all animal experiments were approved by the Animal Care and Use Committee of Shanghai Jiao Tong University. All animal experiment procedures followed the rules of the Committee on Animal Care of Shanghai, China and the ARRIVE guidelines.

**Consent for publication**

Not applicable.

**Availability of data and materials**

Raw data of sequencing is available at the National Omics Data Encyclopedia (NODE, https://www.biosino.org/node/, Project ID: OEP001917) [68].

**Competing interests**
We declare no competing interests.

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**Authors’ contributions**

NW and WQ performed the experiments, collected and analyzed the data, and wrote the article. NW and ZZ prepared biological samples, and performed the experiments. NW and MZ were responsible for bioinformatics investigation. NW, ZZ, JX, RS, LW, PPX, and SC recruited patients, collected study data, and prepared biological samples. LD and HMX reviewed the histopathologic diagnoses. HF, YCW, CL, XJS and FZ gave technical support. WLZ conceived the study, directed, and supervised research and wrote the manuscript. All authors read and approved the final manuscript.

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Tables
|                              | HBsAg+ FL (n=43) | HBsAg- FL (n=210) | p value |
|------------------------------|------------------|-------------------|---------|
| Age                          | 0.044            |                   |         |
| ≤60 years                    | 36 (83.7)        | 143 (68.1)        |         |
| >60 years                    | 7 (16.3)         | 67 (31.9)         |         |
| Sex                          | 0.458            |                   |         |
| Female                       | 17 (39.5)        | 96 (45.7)         |         |
| Male                         | 26 (60.5)        | 114 (54.3)        |         |
| ECOG performance             | 1.000            |                   |         |
| 0-1                          | 42 (97.7)        | 206 (98.1)        |         |
| ≥2                           | 1 (2.3)          | 4 (1.9)           |         |
| Ann Arbor                    | 0.778            |                   |         |
| I-II                         | 6 (14.0)         | 26 (12.4)         |         |
| III-IV                       | 37 (86.0)        | 184 (87.6)        |         |
| Spleen involvement           | 0.006            |                   |         |
| Yes                          | 18 (41.9)        | 46 (21.9)         |         |
| Normal                       | 25 (58.1)        | 164 (78.1)        |         |
| Serum LDH                    | 0.691            |                   |         |
| Elevated                     | 11 (25.6)        | 60 (28.6)         |         |
| Normal                       | 32 (74.4)        | 150 (71.4)        |         |
| Serum β2-MG                  | 0.174            |                   |         |
| Elevated                     | 12 (27.9)        | 80 (38.1)         |         |
| Normal                       | 31 (72.1)        | 130 (61.9)        |         |
| Histology grade              | <0.001           |                   |         |
| I-II                         | 17 (39.5)        | 150 (71.4)        |         |
| IIIA                         | 24 (55.8)        | 57 (27.1)         |         |
| Unknown                      | 2 (4.7)          | 3 (1.4)           |         |
| Ki67                         | <0.001*          |                   |         |
| Median positivity | 50.0% | 30.0% |

*Mann-Whitney U test was used for comparison, and Fisher’s exact tests were used for the other comparisons. ECOG, Eastern Cooperative Oncology Group; LDH, lactate dehydrogenase; β2-MG, β2-macroglobulin.*

See also Additional file 3: Table S2.

**Figures**
Figure 1

Somatic mutation spectrum in FL. (A) Number and types of non-silent somatic mutations identified in FL (n=100). (B) Percentage of non-silent somatic SNVs identified in FL (n=100). (C) Gene mutations mostly related to the pathogenesis of FL identified by WES (n=87) and WGS (n=13). The number and prevalence of mutations are indicated at the right. Lower graph indicates age, Ann Arbor stage, histology grade, and BCL2 translocation. SNVs: single nucleotide variations. See also Additional file 2: Figure S1.
Figure 2

Genetic alterations in HBsAg+ FL and HBsAg- FL. (A) Prevalence of indicated genetic mutations in HBsAg+ FL and HBsAg- FL. Lower graph indicates p value comparing different prevalence in two groups, number of cases, functional clusters, and gene names. (B) Prevalence of genetic mutations according to four major functional clusters in HBsAg+ FL and HBsAg- FL. Prevalence of functional clusters and p values comparing different prevalence in two groups are indicated at the right. (C) Prevalence of BCL2 translocation and BCL2 protein in HBsAg+ FL and HBsAg- FL. Upper graph indicates p values comparing different prevalence in two groups. P values in (A), (B), and (C) are calculated with Fisher’s exact test. See also Additional file 2: Figure S2, and Additional file 3: Table S3, Table S4, and Table S5.
Figure 3

CD8+T- and Th1-enriched phenotype in HBsAg+ FL. (A) GO terms performed on the 1514 DEGs between HBsAg+ FL and HBsAg- FL. (B) IPA revealed predicted upstream regulators that were activated (positive z score) and inhibited (negative z score) in HBsAg+ FL than HBsAg- FL. Right graph indicates log10 (p values) comparing different prevalence in two groups. (C) Normalized expression of MHC I molecules in HBsAg+ FL and HBsAg- FL. Each dot represents a patient and p values comparing different expression in...
two groups are indicated on the top. (D) Normalized expression of MHC II molecules in HBsAg+ FL and HBsAg- FL. Each dot represents a patient and p values comparing different expression in two groups are indicated on the top. (E) Normalized expression of IFN signatures in HBsAg+ FL and HBsAg- FL. Each dot represents a patient and p values comparing different expression in two groups are indicated on the top. (F) Summary radar plot, with 0.5 radial intervals depicting normalized average scores of T-cell subsets in HBsAg+ FL (orange polygon) and HBsAg- FL (blue polygon). (G) Heatmap depicting the expression of genes of CD8+ naïve T cell, CD8+ Tcm, CD8+ Tem and Th1 cells in HBsAg+ FL and HBsAg- FL. Each column represents a patient. GO: Gene Oncology; DEGs: differential expressed genes; IPA: ingenuity pathway analysis; MHC: major histocompatibility complex; CD8+Tcm: CD8+ central memory T cell; CD8+Tem: CD8+ effector memory T cell; Treg: regulatory T cell. P values in (C), (D), (E), and (F) are calculated with two-sided unpaired t-test. See also Additional file 2: Figure S3, and Additional file 3: Table S6, Table S7, and Table S8.

Figure 4
Post-GC B phenotype in HBsAg+ FL. (A) Summary radar plot, with 0.4 radial intervals, depicting normalized average scores of B-cell subsets in HBsAg+ FL (orange polygon) and HBsAg- FL (blue polygon). (B) Heatmap depicting the expression of genes of naïve B cell and switched MBC in HBsAg+ FL and HBsAg- FL. Each column represents a patient. (C) Normalized expression of transcription factors (left panel) and surface markers (right panel) of B cells involved in GC development in HBsAg+ FL and HBsAg- FL. Lower graph indicates transcription factors (left panel) or surface markers (right panel), p values comparing different prevalence in two groups, GC stage, as well as the expression of transcription factors and surface markers at different GC stages according to the literature. (D) Positivity of BCL6, MUM1 and CD10 proteins in HBsAg+ FL and HBsAg- FL. Right graph indicates positivity of proteins and p values comparing different prevalence in two groups. (E) Prevalence of cell-of-origin in HBsAg+ FL and HBsAg- FL. Upper graph indicates p values comparing different prevalence in two groups. Switched MBC: class-switched memory B cell; GC: germinal center; ABC-like: activated B cell-like; GCB-like: germinal center B cell-like. Data in (C) are represented as mean ± SD. And p values in (A) and (C) are calculated with two-sided unpaired t-test. P values in (D) and (E) are calculated with Fisher’s exact test. See also Additional file 2: Figure S4 and Additional file 3: Table S8.

**Figure 5**

Biological function of TNFAIP3 mutation in FL. (A) SC-1 cell viability when transfected with TNFAIP3 wt, L147fs, and R706X. P values (red) comparing different cell viability in TNFAIP3 L147fs and TNFAIP3 wt...
and p values (green) comparing different cell viability in TNFAIP3 R706X and TNFAIP3 wt. (B-C) Percentage of CD3+CD8+T (B, left panel) and Th1 (C, left panel) cells when co-cultured with SC-1 cells transfected with TNFAIP3 wt, L147fs, and R706X. MFI of TIGIT on co-cultured CD3+CD8+T cells (B, right panel) and VISTA on co-cultured Th1 cells (C, right panel). (D) IC50 of SC-1 cells transfected with TNFAIP3 wt, L147fs, and R706X upon lenalidomide treatment at 48h and 72h. (E) Fold change of SC-1 cells transfected with TNFAIP3 wt, L147fs, and R706X when co-cultured with PBMCs upon lenalidomide treatment. (F-G) Fold change of CD3+CD8+T cells (F, left panel) and Th1 cells (G, left panel) when co-cultured with SC-1 cells upon lenalidomide treatment. Fold change of and MFI of TIGIT on co-cultured CD3+CD8+T cells (F, right panel) and VISTA on co-cultured Th1 cells (G, right panel) upon lenalidomide treatment. (H) Survival of zebrafish xenograft models injected with SC-1 cells transfected with TNFAIP3 wt, L147fs, and R706X without (upper panel) and with lenalidomide treatment (lower panel). P values (red) comparing different survival of TNFAIP3 L147fs and TNFAIP3 wt, and p values (green) comparing different survival of TNFAIP3 R706X and TNFAIP3 wt. MFI: mean fluorescence intensity; PBMCs: peripheral blood mononuclear cell. Assays in (A), (B), (C), (D), (E), (F), and (G) are set up in triplicate. Data in (A), (B), (C), (D), (E), (F), and (G) are represented as mean ± SD. P values in (A), (B), (C), (D), (E), (F), and (G) are calculated with two-side unpaired t-test. P values in (H) are calculated with log rank test.

Figure 6

Clinical outcome of HBsAg+ FL upon rituximab-based immunochemotherapy or R2 regimen. (A) Normalized scores of CD8+T cells, TIGIT expression, Th1 cells, and VISTA expression in FL patients with and without HBV-associated mutations of immune modulators (TNFAIP3/CD70/CXCR4/PIM1/KLF2/FAS/CD58/CD83 mutant). Each dot represents a patient and upper graph indicates p values comparing different levels in two groups. (B) Progression-free survival (PFS)
(left panel) and overall survival (OS) (right panel) in HBsAg+ FL and HBsAg- FL patients upon rituximab-based immunochemotherapy treatment (n=175). Upper graph indicates p values comparing different survival in two groups. (C) Progression-free survival (PFS) (left panel) and overall survival (OS) (right panel) in HBsAg+ FL and HBsAg- FL patients upon R2 regimen treatment (n=78). Upper graph indicates p values comparing different survival in two groups. P values in (A) are calculated with two-sided unpaired t-test. P values in (B) and (C) are calculated with log-rank test.

**Supplementary Files**

This is a list of supplementary files associated with this preprint. Click to download.

- Additionalfile1Supplementarymethods.docx
- Additionalfile2Supplementaryfigures.docx
- Additionalfile3SupplementaryTables.xls