METABOLIC, ENDOCRINE, AND GENITOURINARY PATHOBIOLOGY

RNA Expression Profiling of Lymphoepithelioma-Like Carcinoma of the Bladder Reveals a Basal-Like Molecular Subtype

Ujjawal Manocha,^{*} Jordan Kardos,^{*} Sara Selitsky,^{*} Mi Zhou,^{*} Steven M. Johnson,[†] Cori Breslauer,[†] Jonathan I. Epstein,[‡] William Y. Kim,^{*§¶} and Sara E. Wobker^{*†}

From the UNC Lineberger Comprehensive Cancer Center,^{*} and the Departments of Pathology and Laboratory Medicine,[†] Medicine,[§] and Genetics,[¶] University of North Carolina at Chapel Hill, Chapel Hill, North Carolina; and the Departments of Pathology, Urology and Oncology,[‡] Johns Hopkins Hospitals, Baltimore, Maryland

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Address correspondence to Sara E. Wobker, M.D., M.P.H., 303 Brinkhous-Bullitt Bldg., CB#7525, Chapel Hill, NC 27599. E-mail: sara_wobker@med.unc.edu.

Lymphoepithelioma-like carcinoma of the bladder (LELC-B) is a rare subtype of urothelial carcinoma consisting of undifferentiated epithelial cells within a dense inflammatory cell infiltrate. We set out to molecularly characterize LELC-B through RNA expression profiling as well as immunohistochemistry (IHC) to understand its underlying biology. Sixteen cases of LELC-B were identified at Johns Hopkins University. RNA sequencing was performed on 14 cases. IHC staining for programmed cell death ligand 1 (PD-L1) and mismatch repair proteins MutL homolog 1 (MLH1), MutS homolog 2 (MSH2), MSH6, and PMS1 homolog, mismatch repair system component 2 (PMS2) was performed. Transcriptomic profiling of LELC-B showed that they are enriched in a basal-like phenotype, with 12 of 14 LELC-B cases correlating to the basal centroid of the bladder cancer analysis of subtypes by gene expression 47 (BASE47) predictive analysis of microarrays (PAM) classifier. Gene signature analysis confirmed the lymphocyte infiltration profile consistent with the histomorphology. LELC-B lacked features to explain the robust lymphocytic infiltrate, such as loss of mismatch repair protein expression or expression of Epstein-Barr virus transcripts. Nonetheless, PD-L1 IHC was positive in 93% of LELC cases. Our study demonstrates that LELC-B tumors are enriched in a basal-like molecular subtype and share a high level of immune infiltration and PD-L1 expression, similar to basal tumors. The basal-like phenotype is consistent with the known sensitivity of LELC-B to chemotherapy and suggests that immune checkpoint therapy should be explored in this rare disease. (*Am J Pathol* 2020, 190: 134–144; <https://doi.org/10.1016/j.ajpath.2019.09.007>)

Lymphoepithelioma-like carcinoma of the bladder (LELC-B) is a rare variant of urothelial carcinoma composed of sheets or nests of undifferentiated cells with ill-defined cytoplasmic borders and large pleomorphic nuclei with prominent nucleoli. The tumor cells are present within a dense infiltrate of predominantly lymphocytes, plasma cells, and other inflammatory cells.^{1,2} This entity was first described in 1991, and it represents just 0.3% to 1.4% of all bladder carcinomas.³ The histologic appearance of LELC-B closely resembles lymphoepithelioma of the nasopharynx, which is defined by both its histologic appearance and presence of Epstein-Barr virus (EBV) positivity. In bladder, LELC was first shown to be EBV negative via absence of EBV staining by *in situ* hybridization to EBV-encoded RNA (EBER1).⁴ It remains uncertain what

factor is promoting the immune infiltration of these EBV-negative tumors.

Pure or predominant LELC-B (>50% LELC-B features)¹ has shown better overall survival compared with tumors displaying <50% LELC, but the mechanism for this finding is not yet understood. On the basis of a systematic review of LELC-B in the literature, Yoshino et al⁵ reported an overall

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cause-specific survival rate of 83% for LELC-B, with 97% and 95% survival in pure and predominant patterns, respectively, compared with 15% survival in those patients with only focal LELC-B pattern.

Another histologic analog of LELC-B is the medullary phenotype of colorectal carcinoma. These colon tumors show minimal to no glandular growth and are composed of sheets and nests of epithelial cells that have large, pleomorphic nuclei and prominent nucleoli, embedded within a dense lymphocytic infiltrate.⁶ Interestingly, the medullary phenotype of colorectal carcinoma is strongly associated with a high degree of microsatellite instability, showing loss of DNA mismatch repair (MMR) proteins.⁷ Despite this loss, these tumors are often localized and have a better prognosis than other colorectal cancer subtypes.⁸ In addition, tumors with a high degree of microsatellite instability are often found to express programmed cell death ligand 1 (PD-L1), which may be a direct result of tumor infiltrating lymphocytes.⁹

The expression of PD-L1 on tumor cells interacts with its ligand to inhibit T-cell activation. Recently, PD-L1 expression has been the focus of studies evaluating its use as a marker of response on tumor cells and tumor-infiltrating lymphocytes. Consequently, it is believed that the overexpression of PD-L1 leads to worse outcomes in numerous tumor types, including urothelial carcinoma.¹⁰ PD-L1 expression in tumor cells has been associated with a worse disease-specific and overall survival.¹¹

High-grade, muscle invasive bladder cancer demonstrates clinically relevant molecular subtypes based on RNA expression.^{12–16} Although the precise number of subtypes has yet to be determined, there is broad agreement that overarching basal and luminal subtypes exist.¹⁷ The basal, luminal, luminal-infiltrated, and claudin-low molecular subtypes of urothelial carcinoma have emerged as important prognostic markers and may predict response to neoadjuvant chemotherapy.^{13,18} Overall survival appears better in luminal tumors, whereas better response to neoadjuvant chemotherapy in basal tumors leads to improvements in survival over surgery alone.¹⁸ Conversely, claudin-low tumors appear to have a worse prognosis compared with the other subtypes, regardless of neoadjuvant therapy.¹⁸

The molecular and immunohistochemical (IHC) profiles of urothelial cancers with variant histology are beginning to yield important results. Micropapillary urothelial carcinoma is a particularly aggressive variant and has been assigned a luminal subtype, has unique miRNA expression patterns, and demonstrates high rates of *ERBB2* (*HER2*) amplification and activating mutation.^{19,20} Another aggressive variant, plasmacytoid urothelial carcinoma, shows near universal loss-of-function mutations in *CDH1*, which codes for the cell adhesion protein E-cadherin.²¹ This loss is thought to explain the tumor's tendency for aggressive spread beyond fascial planes. Partly because of the rarity of LELC-B, molecular characterization of LELC-B has not been explored. There are potential treatment implications to understanding the molecular

subtypes of these rare variants, given that large-scale clinical trials are not feasible in this population.

Our aim was to profile LELC-B via immunohistochemistry and RNA sequencing. We investigated the immunohistochemical expression of PD-L1 and MMR proteins MLH1, MSH2, MSH6, and PMS2, as well as use PAM classifiers to classify LELC-B into the BASE47 and University of North Carolina at Chapel Hill (Chapel Hill, NC) subtypes of bladder cancer. We hypothesized that LELC-B shows enrichment in markers of the basal molecular subtype of bladder cancer because of the sensitivity to chemotherapy seen in LELC-B. Immune gene signature analyses were done to address the high immune infiltration in LELC-B.

Materials and Methods

LELC-B Case Selection

The study was performed with approval of the Institutional Review Boards at both University of North Carolina at Chapel Hill and Johns Hopkins (Baltimore, MD). Cases were identified by a search of the Johns Hopkins Pathology Lab Information System for cases containing the terms lymphoepithelioma-like carcinoma and bladder from January 1, 2000, to July 25, 2016. Inclusion criteria included patients with a surgical resection specimen from the bladder or transurethral resection of bladder tumor accessioned at Johns Hopkins, with a final diagnosis of LELC-B. Cases with only focal LELC-B were excluded from the study. LELC-B tumors were classified as pure or predominant using the cutoff of >50% for predominant pattern LELC-B and 100% LELC-B for pure pattern.¹ Sixteen cases of pure ($n = 12$) or predominant ($n = 4$) LELC-B were identified in the archives (Table 1). Deidentified, unstained tissue sections were used for IHC and nucleic acid extraction.

Immunohistochemistry

Initial hematoxylin and eosin–stained slides (Figure 4A) were reviewed for tumor adequacy and stained with PD-L1 IHC (Cell Signaling, Boston, MA; E1L3N) and MMR proteins (MLH1, MSH2, MSH6, and PMS2; Cell Marque, Rocklin CA) in a single batch. Additional immunohistochemical stains [cytokeratin 5/6 (CK5/6), CK14, forkhead box A 1 (FOXA1), erythroid transcription factor 3 (GATA3), and uroplakins 2 (UPK2)] were performed by restaining the prior slides used for MMR and one hematoxylin and eosin–stained slide. The IHC staining for these five stains was interpreted independently by two pathologists (S.M.J. and S.E.W.) and any cases not reaching consensus were reviewed together to resolve discrepancies. CK5/6, CK14, FOXA1, GATA3, and UPK2 were given both a stain intensity and percentage of tumor cells staining positively. To develop the modified Allred score, these stains were given a score of 0 to 6 for percentage-positive

tumor cells (0 indicates 0%; 1, 1% to 4%; 2, 5% to 20%; 3, 21% to 40%; 4, 41% to 75%; 5, 76% to 99%; and 6, 100%) and 0 to 3 for stain intensity (0 indicates none; 1, weak; 2, moderate; and 3, strong). Scores were added for a maximum of 9. The H-score was calculated by multiplying the stain intensity by the continuous percentage-positive tumor cells (possible range, 0 to 300)²² (Supplemental Table S1). For MMR IHC, cases were designated as retained expression (any tumor nuclei staining positive at any intensity) or lost expression (negative in all tumor nuclei). For PD-L1, cases were interpreted using the tumor proportion score and combined positive score (CPS) method. CPS was developed for solid tumors stained with the PD-L1 22C3 clone as a way to capture both tumor cell and immune cell staining in one number, calculated as the number of PD-L1–positive cells (tumor cells, lymphocytes, and macrophages) divided by the total number of viable tumor cells, then multiplied by 100.²³ Although the CPS system is not formally validated for PD-L1 clones other than 22C3, it was used to express nontumor cell expression of PD-L1 in these cases.

RNA Extraction and Data Set Processing

Sixteen cases were identified with formalin-fixed, paraffin-embedded tissue available for study. Fourteen cases had sufficient RNA yield after extraction. RNA was extracted with the Roche High Pure RNA Paraffin kit protocol (Roche Diagnostics, Indianapolis, IN). Fourteen samples generated adequate yield for the library preparation with the Illumina TruSeq RNA Access Library Prep Kit (Illumina, San Diego, CA) and were sequenced on an Illumina NextSeq500. FASTQ files from all cases in The Cancer Genome Atlas (TCGA) bladder cancer (TCGA BLCA) data set were obtained from database of Genotypes and Phenotypes (dbGap;

Table 1 Clinical Characteristics of LELC-B Tumor Samples

Case ID	Stage	Histology	Specimen
SW1	pT2	Pure	TURBT
SW3	pT2b	Pure	Cystectomy
SW5	pT2	Pure	TURBT
SW6	At least pT1*	Pure	TURBT
SW7	pT2	Pure	TURBT
SW8	pT2	Pure	Ureterectomy
SW9	pT2a	Pure	Cystectomy
SW10	pT2	Pure	TURBT
SW11	pT2	Pure	TURBT
SW12	pT2	Predominant	TURBT
SW13	pT2b	Pure	Cystectomy
SW14	At least pT1*	Predominant	TURBT
SW15	At least pT1*	Pure	TURBT
SW16	At least pT1*	Pure	TURBT
SW17A	pT2	Predominant	TURBT
SW17B	pT2	Predominant	TURBT

*No muscularis propria present for evaluation in TURBT specimen.
ID, identification; LELC-B, lymphoepithelioma-like carcinoma of the bladder; TURBT, transurethral resection of bladder tumor.

<https://www.ncbi.nlm.nih.gov/gap>). TCGA BLCA along with 14 LELC-B samples were analyzed in R version 3.5.2²⁴ (Supplemental Figure S1A).

Normal cases were excluded, and subtype calls were acquired from TCGA group.¹⁵ Counts aligned to Human Genome Release 29 (GRCh38.p12) via STAR (2.5.3a) and quantified via Salmon (0.8.2).^{25–27} Gene expression data for LELC-B (*n* = 14) and TCGA (*n* = 408) were merged via tximport (1.8.0) and biomaRt (2.36.1).^{28,29} The data were filtered for genes that had expression in at least 80% of the samples (*n* = 422). The raw expression data were normalized to account for sequencing depth differences via size factor normalization and were log transformed via the DESeq2 package (1.20.0).³⁰ Resulting data were adjusted for batch effects via Surrogate Variable Analysis (3.28.0) and Q3 normalized (Supplemental Figure S1B).³¹ The merged data set contained gene expression data from LELC-B (*n* = 14) and TCGA BLCA (*n* = 408) aligned to the hg38 genome. Principal component analysis was conducted to check for outliers (Supplemental Figure S2). All data were visualized via the use of ComplexHeatmap (1.20.0) and ggplot2 (3.1.0) vignettes.^{32,33}

Molecular Characterization

The merged LELC-B–TCGA BLCA data set (*n* = 422) was classified into basal, luminal, or claudin-low subtypes via the BASE47 and the BCL40 PAM Classifier (Supplemental Table S2).^{14,34,35} The Fisher exact test was used to determine significant associations for the BASE47 classifier. Expression data were Z-normalized, and the Pearson distance was used for hierarchical clustering.³² An unsupervised clustering heat map of the bladder cancer classification markers was generated.¹⁵ Gene signatures corresponding to an immune infiltrated tumor subtype via B cells and T cells were investigated in LELC-B.³⁶ Significance was tested by Wilcoxon-Mann-Whitney rank sum method as it does not assume a parametric distribution. Further characterization of LELC-B was performed via a comparison to Urothelial Carcinoma via Gene Set Enrichment Analysis and the MSigDB Hallmark Gene Set Collection^{37,38} (Supplemental Figure S3B and Supplemental Table S3). Just for the Gene Set Enrichment Analysis, three cases were removed from TCGA BLCA data set (one case each for adenomas/adenocarcinomas, epithelial neoplasm not otherwise specified, and squamous cell neoplasm) to yield a data set of *n* = 405 urothelial carcinoma samples.

Detection of Epstein-Barr Virus

Epstein-Barr virus (human herpesvirus 4/EBV) was detected using VirDetect.³⁹ VirDetect aligns reads against a curated and masked viral genome database composed of 1893 vertebrate virus reference genomes from GenBank via STAR RNA-sequencing aligner.²⁶ All reads in the sample FASTQ file that did not align to the hg38 genome were aligned to the viral

genome collection. Our LELC samples were assessed for wild-type EBV (accession identifier: NC_007605.1) and EBV type 2 (accession identifier: NC_009334.1) along with cell lines with validated EBV presence.⁴⁰ The alignments of the tumor sequence to the viral genomes were verified with human endogenous retrovirus as a positive control (accession identifier: NC_022518.1). All sequences used can be referenced via the accession numbers through the National Center for Biotechnology Information website (<https://www.ncbi.nlm.nih.gov/nuccore>).

Results

LELC-B Is Enriched in Markers Corresponding to Basal-Like Bladder Cancer

High-quality RNA was obtained from 14 pure or predominant LELC-B tumors, most of which initially presented with muscle invasion (Table 1). Examination of the LELC-B tumors by BASE47 showed that most of LELC-B has a significant correlation toward the basal centroid, with 12 LELC-B tumors classified as basal-like and 2 LELC-B tumors being classified as luminal-like bladder cancer (Fisher exact $P = 0.0117$) (Figure 1A and Supplemental Table S2). Eight of 10 cases of pure LELC-B and four of four predominant cases correlated to the basal centroid. The PAM classification results were confirmed by unsupervised clustering on the genes that highlight the subtypes within muscle invasive bladder cancer (Figure 2A).¹⁵

A subset of basal tumors have a claudin-low phenotype akin to the breast cancer claudin-low subtype.^{34,41} It was determined whether any of the basal-like LELC-B tumors showed an enrichment in markers of the claudin-low subtype of bladder cancer using the previously published BCL40 PAM Classifier.³⁴ None of the LELC-B basal tumors were characterized as the claudin-low subtype (Figure 1B). Therefore, although LELC-B tumors exhibit a basal phenotype, they are not claudin-low. The hallmark genes that are representative of bladder cancer subtypes, such as *GATA3* and *FOXA1*, confirm that LELC-B is significantly different from the luminal and claudin-low profile (Figure 2B) and most in keeping with a basal-like subtype.

LELC-B Phenocopies Basal Bladder Cancer via Immune Gene Signatures

The expression of a panel of immune gene signatures that have previously been shown to be representative of differing components of the immune system was examined next.^{36,42} Representative Z-scores of immune gene signatures in a heat map supervised by University of North Carolina at Chapel Hill subtype and rank ordered within each subtype were used (Supplemental Figure S3A).^{36,42,43} Although the LELC-B tumors overall appeared to have heterogeneous expression levels of these immune gene signatures, in

Figure 1 **A:** Waterfall plot for the BASE47 PAM classification of lymphoepithelioma-like carcinoma of the bladder (LELC-B) compared with The Cancer Genome Atlas bladder cancer (TCGA BLCA) data set consisting of muscle-invasive tumors.¹⁴ LELC-B tumors were significantly correlated to the basal centroid (Fisher exact test; $P = 0.0117$). **B:** Waterfall plot for BCL40 PAM classifier.³⁴ LELC-B is not associated with the claudin-low tumor subtype. $n = 408$ (A).

general, LELC-B tumors showed a profile of active immune infiltration. To verify this, the expression of T-cell, B-cell, and an immunosuppression signature was specifically examined. In all three signatures, LELC-B tumors showed gene signature levels similar to basal urothelial carcinoma tumors, yet significantly higher and lower than luminal and claudin-low tumors, respectively (Figure 3). These findings are consistent with the notion that LELC-B tumors have RNA expression patterns similar to basal-like bladder tumors.

LELC-B Has High Tumor Cell PD-L1 Expression

To further classify the immune landscape of LELC-B, immunohistochemistry was performed for PD-L1 (Figure 4B). In 15 of 16 total cases (one case was excluded because of inadequate tissue), 14 (93%) were determined to have positive tumor cell staining for PD-L1 (nine demonstrating high expression and five demonstrating low

A **B**

Figure 2 **A:** Unsupervised clustering of the merged data set on genes characteristic of muscle-invasive bladder cancer.¹⁵ Pearson correlation and average linkage were used on Z scores for the 47 genes. **B:** Supervised clustering on normalized expression counts of *FOXA1* and *GATA3* within the University of North Carolina at Chapel Hill (UNC) subtype scheme. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$ (Wilcoxon–Mann-Whitney rank sum test). ECM, extracellular matrix; EMT, epithelial-mesenchymal transition; LELC, lymphoepithelioma-like carcinoma; LELC-B, LELC of the bladder; TCGA, The Cancer Genome Atlas.

Figure 3 Box plots for gene signature Z scores corresponding to B-cell activation, T-cell activation, and immunosuppression.^{34,36} Lymphoepithelioma-like carcinoma of the bladder (LELC-B) is significantly different from luminal and claudin-low subtypes, but not basal bladder cancer. ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$ (Wilcoxon–Mann–Whitney rank sum test).

expression) (Table 2). The five cases with low expression were all pure LELC-B, whereas the nine cases with high expression included seven pure and two predominant types. The single PD-L1–negative case was a predominant LELC-B. There was no significant association between pure or predominant LELC-B cases and PD-L1 IHC status via Fisher exact test ($P = 0.505$). According to the CPS interpretation, all 14 LELC-B tumors that expressed PD-L1 scored ≥ 10 , which is positive overall. The IHC images demonstrate PD-L1 expression in LELC-B tumor cells, with lack of expression in the infiltrating lymphocytes (Figure 4B).

LELC-B Has Intact MMR Protein Expression

All 16 cases of LELC-B uniformly retained MMR protein expression, the only exception being the loss of MSH6 in a single case of a pure pattern LELC-B tumor. Figure 4, C–F, depicts photomicrographs demonstrating representative cases for each IHC marker: MLH1, MSH2, PMS2, and MSH6. There were no significant associations between MMR intact status and PD-L1 expression. Therefore, although MMR deficiency may explain the robust immune

infiltrate in medullary colorectal carcinoma, LELC-B does not appear to be MMR deficient, at least when assessed by immunohistochemistry.

LELC-B Does Not Associate with Epstein-Barr Viral Co-Infection

A final potential explanation for the robust immune infiltrate associated with LELC-B is the expression of a pathogenic virus (ie, EBV). Although previous studies have shown that LELC-B tumors do not express EBV by EBER staining, this question was asked in an orthogonal manner by querying the RNA-sequencing data for expression of the EBV genome.⁴ To examine EBV presence, reads were aligned to a masked and curated database of vertebrate genomes from GenBank (Materials and Methods). Alignment to human herpesvirus 4 genome (EBV) sequence showed a lack of EBV transcripts in LELC-B sequencing reads (Table 3). As expected, counts aligning to human endogenous retrovirus K113 were present in all samples tested, validating its use as a positive control. RNA expression from known EBV expressing cell lines was included to confirm that this method detects EBV

Figure 4 **A:** Hematoxylin and eosin (H&E)-stained section of lymphoepithelioma-like carcinoma of the bladder, showing large epithelioid cells in a mixed lymphoid background. **B:** Programmed cell death ligand 1 (PD-L1) immunohistochemical (IHC) staining demonstrating high positive staining. **C–F:** MLH1, MSH2, PMS2, and MSH6 IHC all demonstrating retained staining. Scale bars: 200 μ m (**A**); 100 μ m (**B–F**).

transcripts (Table 3). These results are consistent with prior studies that used *in situ* hybridization to *EBER1* to detect EBV presence and support in an orthogonal manner that LELC-B tumors do not harbor EBV.

Discussion

Histologic variants of urothelial carcinoma are relatively rare, with approximately 15% to 25% of bladder cancers containing morphologic patterns distinct from conventional urothelial carcinoma.^{44,45} The World Health Organization specified these variants in their 2016 monograph, and the consistent reporting of variant histology has been encouraged by all pathologists.⁴⁶ An understanding of urothelial variants is critical for diagnostic, prognostic, and therapeutic reasons.⁴⁷ Many of these variants have distinct natural histories, with micropapillary, plasmacytoid, and sarcomatoid variants associated with poorer outcomes. To our knowledge, this is the first study investigating the molecular

characterization and immune profiling of LELC of the bladder.

Molecular characterization of high-grade bladder cancers, specifically intrinsic molecular basal and luminal phenotypes, is increasingly recognized as prognostic and predictive biomarkers.^{12–14} For example, multiple groups have demonstrated that the luminal subtype has a significantly better overall survival with or without neoadjuvant chemotherapy than the basal and claudin-low subtypes.¹⁸ In addition, one study has suggested that the basal subtype may be more responsive to platinum-based chemotherapy.¹⁸ Finally, because of differential immune infiltration, the subtypes might also be primed for immune checkpoint inhibitor response, although this has yet to be confirmed. On the basis of these observations and others, some groups have molecularly subtyped both variant and divergent histologic subtypes of bladder cancer in hopes of better understanding the underlying biology and potential implications for treatment choice.

Using the BASE47 gene set predictor, LELC-B cases show a basal-like phenotype. The basal subtype is so termed

Table 2 Summary of LELC-B Immunohistochemistry Results

Characteristic	LELC-B cases
<i>n</i>	16
PD-L1 staining (IHC), <i>n</i> (%)	
High	9 (60)
Low	5 (33)
No expression	1 (7)
MMR deficiency (IHC), <i>n</i> (%)	
MutL homolog 1 (MLH1)	0 (0)
MutS homolog 2 (MSH2)	0 (0)
MutS homolog 6 (MSH6)	1 (6)
PMS1 homolog 2 (PMS2)	0 (0)

IHC, immunohistochemistry; LELC-B, lymphoepithelioma-like carcinoma of the bladder; MMR, mismatch repair; PD-L1, programmed cell death ligand 1.

because of its increased expression of basal keratin markers (KRT5, KRT6, and KRT14), leading to a more basaloid/stem cell phenotype.¹⁴ Although the basal subtype of bladder cancer confers a worse prognosis overall, it appears to show increased sensitivity to chemotherapy compared with non-basal subtypes.¹⁸ LELC-B is historically thought to be exquisitely sensitive to chemotherapy and, although merely correlative, the enrichment of LELC-B in the basal subtype is at least consistent with this chemoresponsive phenotype.

Hematoxylin and eosin stains of LELC demonstrate high lymphocytic infiltrate, and previous work has shown that the lymphocytes are predominantly CD8⁺ and CD4⁺ T cells.² In our studies, the immune gene signatures were also broadly up-regulated in LELC-B and had levels comparable to the basal-like urothelial carcinoma. Investigation of

mechanisms underlying this robust lymphocytic infiltrate suggested no evidence for loss of MMR proteins or the presence of active EBV transcription. Unfortunately, tumor mutational burden and neoantigen load could not be assessed as DNA sequencing could not be performed on the LELC-B samples. Therefore, although these studies confirm the presence of an immune infiltrate within the tumor microenvironment, they do not elucidate the underlying drivers of this phenotype or define the relative clonality of the T cell. Nonetheless, it is tempting to speculate that the lymphocytic infiltrate may represent a robust, but exhausted, antitumor immune response that could be reinvigorated through immune checkpoint therapy.

Immune checkpoint inhibitors are being investigated as second-line therapy after platinum-based chemotherapy in metastatic bladder cancer. In the IMvigor210 clinical trial, atezolizumab, an anti-PD-L1 antibody demonstrated a correlation between PD-L1 expression on tumor-infiltrating lymphocytes with longer overall survival.⁴⁸ According to the PD-L1 IHC results, immune cells in LELC-B do not stain for PD-L1 (Figure 4B). However, the CheckMate275 trial showed that nivolumab, an antibody to programmed cell death protein 1 (PD-1), led to response regardless of high or low PD-L1 CPS scores in metastatic urothelial carcinoma.⁴⁹ Similarly, pembrolizumab, a PD-1 antibody, showed clinical benefit but was not associated with high PD-L1 CPS.⁵⁰ With immune checkpoint blockade becoming a prominent second-line therapy, further research is needed to investigate the dense tumor lymphocytic infiltrate seen histologically in LELC-B and any associations with better prognosis of pure and predominant LELC-B.⁵

Table 3 Viral Genome Quantification through STAR, including EBV (HHV-4)²⁸

Case ID	Human_endogenous_ retrovirus_K113_complete_ genome_unmasked	Human_herpesvirus_ 4_complete_wild_ type_genome	Human_ herpesvirus_4	Human_ immunodeficiency_ virus_1	Simian_ virus_40
SWLELC10	212	0	0	0	0
SWLELC12	146	0	0	0	0
SWLELC13	2	0	0	0	0
SWLELC15	632	0	0	2	0
SWLELC16	124	0	0	0	0
SWLELC17A	220	0	0	0	0
SWLELC17B	90	0	0	0	0
SWLELC1	1016	0	0	0	0
SWLELC3	94	0	0	0	0
SWLELC4	58	0	0	0	0
SWLELC6	402	0	0	0	0
SWLELC7	46	0	0	42	8
SWLELC8	14	0	0	0	0
SWLELC9	902	0	0	0	0
Daudi	126	38,316	33,734	0	0
GRANTA-519	206	89,142	78,894	0	0
SNU-719	288	23,984	25,384	0	0

Lymphoepithelioma-like carcinoma of the bladder does not contain sequence aligning to EBV, which is concurrent with previous studies.^{2,4} RNA-sequencing data for cell lines validated to contain EBV were obtained from Klijn et al.⁴⁰

EBV, Epstein-Barr virus; HHV-4, human herpesvirus 4; ID, identification.

The limitations of our study are inherent in the use of formalin-fixed, paraffin-embedded archival tissue for RNA analysis. Biases may be present in data as RNA extracted from formalin-fixed, paraffin-embedded slides has lower yields and integrity than flash frozen tissue RNA. The data set processing removes any technical batch effects and minimizes bias. In any future studies, immune cell PD-L1 levels specifically should be investigated to determine whether LELC-B could be a candidate for immune checkpoint inhibitors. Further explanation for the robust immune infiltration should also be examined, such as the somatic mutational load or neoantigen burden. Additional investigation of a larger series of LELC-B cases with clinicopathologic outcomes should be attempted to confirm this study's findings and expand on the results.

In summary, the immune infiltration and immunosuppression profile in LELC is consistent with the low cause-specific mortality rate; however, the immune gene signature expression was lower than expected. With Food and Drug Administration approval of immune checkpoint inhibitors for bladder cancer, the knowledge of the immune profile of the tumor is increasingly pertinent in treatment decision making.⁵¹ The robust lymphocytic infiltrate in LELC may be predictive as immune checkpoint blockade therapies gain Food and Drug Administration approval as second-line therapies for muscle invasive bladder cancers.⁵² Ongoing studies include RNA sequencing and further profiling of LELC-B tumors.

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Author Contributions

S.E.W. designed the study, performed literature search, collected data, and wrote the manuscript; U.M. generated and analyzed data, performed literature search, generated the figures, and wrote the manuscript; J.K. interpreted and analyzed the data; M.Z. analyzed the data; S.J. generated and analyzed data and reviewed the manuscript; C.B. generated and analyzed data; S.S. generated and analyzed data and reviewed the manuscript; W.Y.K. designed the study and reviewed the manuscript.

Supplemental Data

Supplemental material for this article can be found at <http://doi.org/10.1016/j.ajpath.2019.09.007>.

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