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Lymphocyte-Specific Tyrosine Kinase Expression In Ovarian Cancer: A Valuable Prognostic Indicator

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LYMPHOCYTE-SPECIFIC TYROSINE KINASE EXPRESSION IN OVARIAN CANCER: A VALUABLE PROGNOSTIC INDICATOR

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LYMPHOCYTE-SPECIFIC TYROSINE KINASE EXPRESSION IN OVARIAN CANCER: A VALUABLE PROGNOSTIC INDICATOR

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Cellular immune response, specifically tumor infiltrating lymphocytes (TILs), has been correlated to survival in epithelial ovarian cancer; however, specific gene expression patterns for this response remain poorly understood. The objective of this research was to investigate the prognostic and biologic significance of immune-related gene expression in high grade serous ovarian cancer (HGSOC). To do so, a panel of immune related gene expression was evaluated in HGSOC utilizing The Cancer Genome Atlas (TCGA) and validated in an independent cohort of ovarian tumors. Based on the strong association with survival, the cohort was grouped into LCK (lymphocyte specific tyrosine kinase) high and non-LCK high tumors and profiles of gene expression and clinical information were obtained. We demonstrate that mRNA upregulation of LCK was correlated with the strongest improvement in survival of the genes investigated. When compared to previously validated metrics such as cytolytic activity score (CYT), LCK proved to be a more discerning prognosticator across tumor types available in the TCGA. In ovarian cancer, correlated gene enrichments were notable for chemokine and immunoglobin complex related genes, ie B cell related transcripts. Therefore, this research shows that LCK is a biomarker of prognostic and biological

importance, potentially due to its ability to capture the genomic signature of cooperative T and B cell interaction. This provides essential support for further investigation into the role of tumor infiltrating B cells (TIL-B) and tertiary lymphoid structures (TLS), from which insights into this cooperation can be drawn. As ovarian cancer is the leading cause of death from gynecologic malignancy, such insights have the potential to not only offer important prognostic information but also may provide novel therapeutic approaches to the treatment of this deadly disease.

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Immunogenicity of Ovarian Cancer

There is growing evidence to support a pivotal role of the immune system in the pathogenesis of cancer; in high grade serous ovarian cancer (HGSOC) and other cancers the presence of high levels of tumor infiltrating lymphocytes (TILs) has been associated with improved progression free survival (PFS) and overall survival (OS) [1]–[7]. TIL infiltration of treatment naïve tumors was associated with a significantly improved median progression free (22.4 vs 5.8 months, $p < 0.001$) and overall survival (50.3 vs 18.0 months, $p < 0.001$) compared to tumors with no T-cells present [7]. Additionally, expression of alternative markers of activation of the immunoreactivity, including upregulation of programmed-death ligands 1 and 2 (PD-L1 and PD-L2), has also been shown to correlate with improved OS [8]. Recent publications reported a histotype-specific nature of immune infiltration and demonstrated the magnitude of survival benefit in ovarian cancer was dose dependent on CD8 positive TILs [9], [10].

However, the use of TIL classification by immunohistochemistry (IHC) for clinical decision-making currently remains in its early stages, as IHC can be time intensive and requires comparatively specialized pathology input. Additionally, while prognostic ability is useful, ideally biomarkers should also be relevant to predict response to therapy. For example, the use of PDL1 staining has emerged as an intuitive marker for prediction of response to immune checkpoint inhibitors, at least in some cancers. Immune checkpoint inhibitors are a

novel class of drugs which are monoclonal antibodies that block PD-1/PDL-1 and result in increased immune response to tumor. These drugs have revealed efficacy in 10-15% of heavily pretreated ovarian malignancies with some durable responses [11]–[15]. However, given the low response rates and significant toxicities of such therapies, studies aimed at identifying factors to provide more personalized prognostication for response to these therapies in particular are of utmost importance. However, the predictive accuracy of IHC markers to determine response to immune checkpoint therapy for ovarian cancer remains unknown, as many trials remain ongoing and have not yet reported translational endpoints. It is worth mentioning that the reported response rates to PD1/PDL1 targeting drugs are not appreciably higher in clinical trials using PDL1 positivity by IHC as an eligibility criterion [26]. Given the difficulties presented with IHC analyses, investigation into genomic markers represents an exciting potential alternative, but have thus far yielded mixed results.

Genomic Biomarkers in Ovarian Cancer

The biological basis and the identification of reliable genomic markers with prognostic significance have proven elusive. Multiple studies have attempted to identify gene expression signatures and their predictive ability for clinical outcome, including overall survival, time to relapse or response to chemotherapy [16]–[21]. However, gene expression models have thus far been limited by complexity, often requiring large and heterogeneous gene signatures in order to demonstrate prognostic ability. For example, one study using an analysis of 68 HGSOC samples validated a 115 gene signature, termed the Ovarian Cancer Prognostic Profile (OCPP) [21]. When attempting to classify included relevant genes by function, 17

different function groups were required and included both immune-related function, angiogenesis pathways, and cell-cell adhesion signaling related to tumor epithelial-mesenchymal transition. Discrete biologic etiologies for predictive ability is limited with such heterogeneity.

There are very few studies which used unsupervised classification approaches, thus prior classification has previously been subject to inherent bias in grouping determinations. Approaches to such unbiased categorization have been limited by sample size and by inclusion of heterogeneous histologic ovarian tumor subtypes [22]–[24]. Perhaps the most comprehensive of such unsupervised clustering research analyzed 285 samples, including both high grade serous and endometrioid tumors [16]. Optimal clustering of array data revealed six different molecular subtypes, which were clinically relevant as they grouped by histologic subtype and clinical outcome. However, each subtype displayed distinct levels of immune cell infiltration and reactive stroma gene expression signatures, making it difficult to determine driver biologic pathways.

Based on these subtypes, the original publication of the ovarian cancer TCGA analysis attempted to categorize samples into more biologically based functional groups [25]. The investigators identified an “immunoreactive” group as one of the four subtypes of high-grade serous ovarian cancer based on transcriptional profiling. In this analysis, T-cell chemokine ligands, CXCL11 and CXCL10, and the receptor, CXCR3, characterized the immunoreactive subtype. Unfortunately, there was no prognostic impact on survival associated with this

immunoreactive subtype [25]. There is a critical unmet need to establish reliable genomic biomarker(s) for this tumor immune response with utility in prognostication and stratification of untreated ovarian cancers.

Genomic Prognostic Scoring Systems in Other Tumor Types

Investigation of such genomic biomarkers can be informed by research in alternate tumor types and then applied to HGSOC. One well published genomic prognostic feature is the cytolytic activity score (CYT), a quantitative measure of immune cytolytic activity based on transcript levels of perforin (PRF1) and granzyme A (GZMA) [11]. These two molecules reflect the central mechanism for cytotoxic lymphocyte killing; perforin is responsible for the creation of pores within the target cell membrane which then allow for the entry of granzymes that cleave caspases and induce apoptosis. CYT has been shown to be a useful metric of cyototoxic activation and subsequent improved survival in multiple other tumor types [11], [26]–[28].

In pancreatic cancer, a study of expression data from 134 tumors available in The Cancer Genome Atlas (TCGA) revealed that CYT-high tumors exhibit increased expression of multiple immune checkpoint related genes, and, interestingly, were inversely correlated with genomic alterations, indicating that intrinsic oncogenic processes drive immune suppression. However, this analysis did not report a relationship of CYT score subsets to clinical outcome and prognosis [27]. Similarly, an analysis of CYT in colorectal cancer demonstrated that

CYT-high tumors were associated with high levels of activated T-cells but did perform subsequent analysis in order to report improved overall survival in this tumor subset[26].

The definition of cut-off points for CYT-high and CYT-low tumors is not standardized across tumor types. In the above literature regarding pancreatic cancer, CYT was defined as top 10 percentile compared to bottom 25^{th} percentile [27]. In colorectal, the threshold for dichotomization was determined at multiple candidate cut-points and the cut-off point that gave the most significant results was chosen [26]. A meta-analysis, performed across tumor subtypes within the TCGA, including ovarian cancer, found very diverse levels of CYT across different cancer types. Results were not dichotomized or clustered and instead were correlated as a continuous variable to various markers of immunoreactivity, namely immune checkpoint molecules and TILs [28].

CYT score has not been independently studied in ovarian malignancy; further description of this score specifically in HGSOC is needed. Additionally, based on the meta-analysis by Roufas et al, this score can serve as a benchmark against which other proposed genomic biomarkers can be evaluated.

Preliminary Data

To address this unmet need to establish genomic biomarkers in ovarian malignancy, we undertook a preliminary study in which we analyzed 535 high grade serous ovarian samples in the TCGA dataset using the cBioPortal platform, 520 of which had Affymetrix U133

microarray data available for mRNA analysis [25], [29], [30]. Analysis of the TCGA was performed investigating the upregulation of a panel of immune related genes including: CD3E, CD3D, CD2, CD4, Perforin 1 (PRF1), Granzyme A (GZMA), CD19, and CD20 (MS4A1) and LCK (**Figure 1**). CD8A data was unavailable within the microarray. Progression free and overall survival data were collected for each of the above genes and compared in elevated and non-elevated samples.

LCK (lymphocyte specific tyrosine kinase) was shown to have the strongest association with survival; patients with high LCK mRNA expression had a median progression free survival of 29.4 months, compared to 16.9 in those without high LCK expression (p=0.003**)**. Patients with high LCK had significantly longer overall survival than non-LCK high with median overall survival time of 95.1 months and 44.5 months, respectively (p= 0.001). Only two other markers chosen were statistically significantly associated with survival and shown to have less dramatic prognostic differences. High expression of B-cell marker CD20 (MS4A1) was associated with survival, with median PFS of 27.2 months (p= 0.08) and overall survival of 86.1 months (p=0.02), while CD3E elevation had a significant association with PFS (p=0.016) but was not associated with OS (p=0.330). High expression of the other immune related genes tested above was not associated with survival.

This stringent high criteria for mRNA expression in LCK was found in 23 (4%) of all cases (**Figure 1**). We also evaluated potential demographic, clinical, and pathological differences between LCK high and remaining samples (**Table 1)**. The median age of the entire cohort

was 59 years old (30-89 years), and most patients were advanced stage (72.9 % stage IIIC, 16.0% stage IV). No differences were detected between the two groups with respect to clinical characteristics, including age, race, ECOG performance status, clinical stage, and tumor grade.

These data demonstrate LCK expression has the potential to be a clinically important prognostic indicator in ovarian malignancy. LCK likely broadly captures the immunoreactivity of a tumor and thus is a less heterogeneous biologic marker than those previously studied, which have included both immune pathways as well as cell adhesion signaling and/or angiogenic molecular indicators. This simplicity is valuable as it may be more easily evaluated to frame further biologic hypotheses, especially within the context of HGSOC response to treatment options which rely on this immunoreactivity, such as immune checkpoint inhibitors.

Public Health Significance

High grade serous ovarian cancer (HGSOC) is the leading cause of death from gynecologic malignancy, with over 22,000 cases per year in the United States and over 14,000 deaths [31]. The high mortality rate is due to the fact the majority of ovarian cancer presents at advanced stage III/IV and has a high risk of recurrence despite initial response to traditional platinum based therapy. These patients are treated with a large and ever-expanding amount of healthcare resources such as hospitalizations, surgical treatment, and chemotherapeutic regimens. Additionally, novel treatment options such as immune

checkpoint inhibitors, which leverage the immune-reactive nature of this malignancy, are rapidly expanding in use but are also currently nearly prohibitively expensive [32], [33].

There is no currently available effective screening method for ovarian cancer, thus primary prevention options remain limited [34]. Public health interventions must focus instead on secondary prevention, with early detection and improved prognostication, as well as tertiary prevention to reduce morbidity and recurrence. The identification of relevant clinically applicable biomarkers will allow for better patient counseling regarding prognosis and more educated decision-making regarding treatment planning. Additionally, the potential for selection of treatment based on a biomarker predicted response has the promise to drastically improve both treatment selection and, consequently, treatment efficacy.

The current study contributes meaningfully to this gap in knowledge. As IHC markers of immunogenicity have not yet proved clinically useful, the current study uses the known TIL correlation with survival, to delve more deeply into potential immune related gene expression biomarkers. We capitalize on preliminary data obtained by the current investigator, which identifies LCK as a particularly valuable biomarker. The current study provides essential validation of these findings and better characterization of LCK's utility as compared to previously validated markers such as CYT score.

Hypothesis, Research Question, Specific Aims or Objectives

Tumor infiltrating lymphocytes (TILs) are correlated with better prognosis in high grade serous ovarian cancer (HGSOC); however, specific gene expression patterns for this response

remain poorly understood. There is a critical unmet need to establish such genomic biomarkers within this deadly gynecologic malignancy.

Preliminary data demonstrates LCK correlates with both progression free and overall survival in available TCGA samples. **Therefore, we hypothesized this prognostic ability of LCK would be validated by protein-expression as evaluated by IHC in an independent cohort of HGSOC samples. We additionally hypothesized LCK would prove to be a better predictor of survival than cytolytic activity score (CYT) in HGSOC.**

We investigated these independent hypotheses via the following specific aims:

Aim 1: Validate the association between LCK and survival in an independent cohort of HGSOC samples via immunohistochemistry

Aim 2: Compare the prognostic capability of LCK to previously validated CYT score within the TCGA

METHODS

The high grade serous ovarian cancer (HGSOC) provisional data set from the TCGA was analyzed to explore the correlation between a panel of immune cell markers and clinical outcome [18]. For mRNA expression analysis, Affymetrix U133 microarray data was used and only samples for which these data were available included. Samples were divided into "high expression" and "non-high expression" groups using the Cbioportal web interface, for the following markers: CD2, CD3E, CD3D, CD4, GZMA, PRF1, CD19, MS4A1 and LCK [19], [20] where high expression was defined as expression within the top 3% (1.86 SD). As described in the background, LCK was demonstrated to significantly predict both progression free and overall survival. The current study represents the subsequent analyses of this same data required to validate this finding and further explore the value of LCK as a prognostic biomarker.

Aim 1: Validate the association between LCK and survival in an independent cohort of HGSOC samples via immunohistochemistry

Study Design, Setting, and Study Population:

LCK protein expression was determined via immunohistochemistry on an independent cohort of 72 ovarian cancer samples using a commercially available anti-LCK antibody (HPA003494, Sigma-Aldrich). Additionally, CD8 (T-cell marker) and CD20 (B-cell marker) immunohistochemistry staining was performed in this cohort (CD20:SAB5600082, Sigma-Aldrich, CD8: CD8-4B11-L-CE, Leica Biosystems), and demographics and survival data were abstracted.

Additionally, IHC was performed across a range of benign and malignant serous neoplasms on an available tissue microarray (TMA). The TMA contained a spectrum of serous gynecological tissues, including normal fallopian tube epithelium obtained at the time of salpingo-oophorectomy for benign ovarian cystadenomas and high grade serous carcinomas. It included a total of 20 normal fallopian tube samples, 14 high grade ovarian serous carcinoma tissues, and 13 benign serous cystadenomas. Each tissue specimen was represented as 3 independent cores on the TMA.

Data Collection and Analysis:

A semi-quantitative IHC score was assigned and evaluated by the investigator, with confirmation by pathology collaborators including a senior gynecologic pathologist. LCK status of these samples was unknown, therefore all parties were initially blinded to outcome. For scoring purposes, tissue LCK+ lymphocytes staining was classified as none (0, average of one or less LCK+ lymphocyte), low (1, less than 10 LCK+ lymphocytes), medium (2, greater than 10 but less than 40 LCK+ lymphocytes), and high (3, greater than 40 LCK+ lymphocytes or multiple germinal centers). The same cut offs were used for CD8 and CD20 positivity. The counts were averaged over 3 fields for independent pathology samples or averaged over the 3 cores for TMA samples.

Statistical Analysis

IHC score comparison was performed using the Mann-Whitney U test with $p<0.05$ considered significant. Spearman correlations were performed to assess the strength of association of LCK, CD20, and CD8. Strength of correlations analysis was performed using R version 3.4.1 package "cocor" [21].

The sample size was pre-defined by availability of tumor samples; there were 72 HGSOC samples available for analysis. TCGA data in preliminary analysis demonstrated an improvement in survival from 16.9 months to 29.4 months for those with high LCK expression. Therefore, we assumed a similar doubling of survival in our validation cohort. However, preliminary data used a very stringent definition (top 3%) of high LCK expression, and IHC analysis is unable to have this level of specificity or discriminatory capacity. Therefore, based on prior literature in other tumor types and the known immunogenicity of ovarian malignancy, we estimated that approximately 30% of tumors would be LCK-high using the much less stringent IHC scoring metric. At a significance level of 0.05, with this expected ratio of 30% and assumed doubling of median survival, the 72 samples available provided a 74% power to detect a difference between LCK-high and non-LCK-high tumors.

Human Subject Samples and Data Considerations

Patients included in this study are a subset of patients with HGSOC who sought treatment at The University of Texas MD Anderson Cancer Center (MDACC). Eligibility required pathologic confirmation at MDACC and availability of sample tissue blocks within the

Gynecologic Oncology Tumor Bank. All patient samples were collected on a tissue banking protocol approved by MDACC Institutional Review Board (IRB) (LAB06-0412). The included 72 samples are a previously established sample subset available in the lab of Dr. Samuel Mok, who provided consent for their use for the current project. Clinical information for this subset of patients was also previously collected in Dr. Mok's lab.

The current analysis represents a retrospective IHC analysis of these blocks and correlation with clinical data, without additional patient contact or intervention. Therefore, this study did not involve any additional testing, treatment or biopsy procedures. Retrospectively, it would be impractical to obtain consent from patients who may be lost to follow-up, no longer in treatment or have died, therefore, the current study was performed under a waiver of informed consent.

All data collected is confidential and used only for research purposes. The data resides on the secure, password protected, 21CFR part 11 compliant database. Subjects were not identified by name during data entry and analysis. Subject names do not appear in any report or paper related to the study. Only the investigator and collaborators (including PI, Dr Amir Jazaeri, and Dr. Samuel Mok) have access to the data. Unique study numbers have replaced the HIPAA identifiers in the analytical file.

Aim 2: Compare the prognostic capability of LCK to previously validated CYT score within the TCGA

Study Design, Setting, and Study Population:

Similar to preliminary data, the high grade serous ovarian cancer (HGSOC) provisional data set from The Cancer Genome Atlas was analyzed [18]. For mRNA expression analysis, complete RNA sequencing data, rather than Affymetrix microarray data, was used for analyses to be performed across 30 tumor types available in the TCGA. The following tumor types (project code and n=sample size) were included: adrenocortical carcinoma (ACC, n=92), bladder/urothelial (BLCA, n=412), breast invasive carcinoma (BRCA, n=1098), cervical squamous cell carcinoma and endocervical adenocarcinoma (CESC, n=307), cholangiocarcinoma (CHOL, n=51), colon adenocarcinoma (COAD, n=461), esophageal carcinoma (ESCA, n = 185), glioblastoma multiforme (GBM, n=617), head and neck squamous cell carcinoma (HNSC, n=528), kidney renal clear cell carcinoma (KIRC, n=537), kidney renal papillary cell carcinoma (KIRP, n=291), acute myeloid leukemia (LAML, n=200), low grade glioma (LGG, n=516), liver hepatocellular carcinoma (LIHC, n=377), lung adenocarcinoma (LUAD, n = 585), lung squamous cell carcinoma (LUSC, n=504), mesothelioma (MESO, n=87), ovarian serous cystadenocarcinoma (OV, n=608), pancreatic adenocarcinoma (PAAD, n=185), pheochromocytoma and paraganglioma (PCPG, n=179), prostate adenocarcinoma (PRAD, n=500), rectum adenocarcinoma (READ, n=172), sarcoma (SARC, n=261), skin cutaneous melanoma (SKCM, n=470), stomach adenocarcinoma (STAD, n=443), testicular germ cell tumors (TGCT, n=150), thyroid carcinoma (THCA,

n=507), uterine corpus endometrial carcinoma (UCEC, n=560), uterine carcinosarcoma (UCS, n=57), and uveal melanoma (UVM, n=80).

Data Collection and Analysis:

For this analysis in each cancer the LCK-high expressing population (the top 10%) was compared to the LCK-low population (bottom 10% in expression). The definition of high and low expressing samples was broadened from the stringent top 3% used in the preliminary analysis to make results more generalizable to a broader population of ovarian malignancy.

LCK prognostic capacity was compared to CYT, which has been previously defined [14]. Briefly, to calculate CYT score, total raw read counts per gene were converted to transcripts per million (TPM), which were calculated by dividing by the gene's maximum transcript length to provide a coverage depth estimate and scaling to sum to a total depth of 1e6 per sample. CYT was then calculated as the geometric mean of GZMA and PRF1 expression values in TPM. As dichotomization of CYT-high and CYT-low is nonstandardized across prior literature, we defined high and low CYT groups as top 10% and bottom 10% for comparison, in order to parallel the LCK definition most exactly.

Statistical Analysis

Descriptive statistics (n, percent, mean, standard deviation) were calculated to summarize patient demographics. Cox regression and backwards stepwise regressions were performed to assess overall survival (OS) and progression free survival (PFS) for LCK gene expression

and dichotomized CYT groups. Correction for multiple comparisons was performed using Bonferroni method. Statistical analyses were performed using SAS 9.4 for Windows (SAS Institute Inc., Cary, NC).

Human Subject Samples and Data Considerations

To address this aim, analyses mirrored what was performed to provide the preliminary data. Specifically, the high grade serous ovarian cancer (HGSOC) provisional data set from The Cancer Genome Atlas was analyzed [18]. This is a publicly available dataset that is query-able via the cBioPortal web interface [19], [20]. This cBioPortal for Cancer Genomics was originally developed at Memorial Sloan Kettering Cancer Center (MSK), and is hosted by the Center for Molecular Oncology at MSK. The software is available under an open source license and is maintained by a multi-institutional team, consisting of MSK, the Dana Farber Cancer Institute, Princess Margaret Cancer Centre in Toronto, Children's Hospital of Philadelphia, The Hyve in the Netherlands, and Bilkent University in Ankara, Turkey.

The proposed analysis represents additional analysis of this publically available data, thus does not involve any HIPAA identifiers. No additional consent is required for data usage, apart from appropriate citation of data source in any subsequent manuscript publication.

JOURNAL ARTICLE

Title: Lymphocyte-Specific Kinase Expression is a Prognostic Indicator in Ovarian Cancer and Correlates with a Prominent B-Cell Transcriptional Signature

Journal: Cancer Immunology, Immunotherapy (CII)

Abstract:

Objective: To investigate the prognostic and biologic significance of immune-related gene expression in high grade serous ovarian cancer (HGSOC).

Methods: Gene expression dependent survival analyses for a panel of immune related genes were evaluated in HGSOC utilizing The Cancer Genome Atlas (TCGA). Prognostic value of LCK (lymphocyte specific tyrosine kinase) was validated using immunohistochemistry (IHC) in an independent set of 72 HGSOC. Prognostic performance of LCK was compared to cytolytic score (CYT) using RNAseq across multiple tumor types. Differentially expressed genes in LCK high samples and gene ontology enrichment were analyzed.

Results: High pre-treatment LCK mRNA expression was found to be a strong predictor of survival in a set of 535 ovarian cancers. Patients with high LCK mRNA expression had a longer median progression free survival (PFS) of 29.4 months compared to 16.9 months in those without LCK high expression (p=0.003), and longer median overall survival (OS) of 95.1 months versus 44.5 months (p= 0.001), which was confirmed in an independent cohort by IHC (p=0.04). LCK expression was compared to CYT across tumor types available in the TCGA and was a more significant predictor of prognosis in HGSOC. Unexpectedly, LCK high samples also were enriched in numerous immunoglobulin-related and other B cell transcripts.

Conclusions: LCK is a better prognostic factor than CYT in ovarian and other cancers. In HGSOC, LCK high samples were characterized by higher expression of immunoglobulin and B cell related genes suggesting a cooperative interaction between tumor infiltrating T and B cells may correlate with better survival in this disease.

Introduction

Ovarian cancer is the leading cause of death from gynecologic malignancy, with over 22,000 cases per year in the United States and over 14,000 deaths [1]. The high mortality rate is due to the fact that the majority of ovarian cancer presents at advanced stage III/IV and has a high risk of recurrence despite initial response to traditional platinum based therapy. There is growing evidence to support a pivotal role of the immune system in the pathogenesis of cancer; in ovarian cancer and others the presence of high levels of tumor infiltrating lymphocytes (TILs) has been associated with improved PFS and OS [2]–[8]. However, this impact is in the context of a complex interplay between multiple aspects of the tumor microenvironment, as T cell type, location, and tumor stromal factors have all been shown to modify survival rates [5], [9]–[13].

In the setting of this complexity, there is a need for reliable biomarker(s) with utility in prognostication and stratification of untreated ovarian cancers. One well published genomic prognostic feature is the cytolytic activity score (CYT), a quantitative measure of immune cytolytic activity based on transcript levels of perforin (PRF1) and granzyme A (GZMA)[14]. These two molecules reflect the central mechanism for cytotoxic lymphocyte killing; perforin is responsible for the creation of pores within the target cell membrane which allow for the entry of granzymes that cleave caspases and induce apoptosis. CYT has been shown to be a useful metric of cyototoxic activation and subsequent improved survival in multiple tumor types [14]–[17]. Therefore, the objective of this study was to investigate a

panel of immune-related genes to determine their prognostic ability and compare to these previously validated metrics.

Materials and Methods

TCGA Data Analysis

To explore the correlation between a variety of immune cell markers and clinical outcome, the high grade serous ovarian cancer (HGSOC) provisional data set from the TCGA was analyzed [18]. Because all information from the TCGA is de-identified and publically available, informed consent by the study participants and approval of an ethics committee were unnecessary to perform this portion of the study. For mRNA expression analysis, Affymetrix U133 microarray data was used and only samples for which these data were available included. Samples were divided into "high expression" and "non-high expression" groups using the Cbioportal web interface, for the following markers: CD2, CD3E, CD3D, CD4, GZMA, PRF1, CD19, MS4A1 and LCK [19], [20] where high expression was defined as expression within the top 3% (1.86 SD). Gene expression and enrichment analyses were performed using BRB-ArrayTools (Version 4.5.1) developed by Dr. Richard Simon and the BRB-ArrayTools Development Team. Gene expression analysis was performed with $p<0.001$ cutoff for significance to guard against false discovery due to multiple comparisons and at least two-fold difference in the geometric mean of expression levels.

Subsequent analysis of RNA sequencing data was performed across 30 tumor types available in the TCGA. The following tumor types (project code and n=sample size) were included:

adrenocortical carcinoma (ACC, n=92), bladder/urothelial (BLCA, n=412), breast invasive carcinoma (BRCA, n=1098), cervical squamous cell carcinoma and endocervical adenocarcinoma (CESC, n=307), cholangiocarcinoma (CHOL, n=51), colon adenocarcinoma (COAD, n=461), esophageal carcinoma (ESCA, n = 185), glioblastoma multiforme (GBM, n=617), head and neck squamous cell carcinoma (HNSC, n=528), kidney renal clear cell carcinoma (KIRC, n=537), kidney renal papillary cell carcinoma (KIRP, n=291), acute myeloid leukemia (LAML, n=200), low grade glioma (LGG, n=516), liver hepatocellular carcinoma (LIHC, n=377), lung adenocarcinoma (LUAD, n = 585), lung squamous cell carcinoma (LUSC, n=504), mesothelioma (MESO, n=87), ovarian serous cystadenocarcinoma (OV, n=608), pancreatic adenocarcinoma (PAAD, n=185), pheochromocytoma and paraganglioma (PCPG, n=179), prostate adenocarcinoma (PRAD, n=500), rectum adenocarcinoma (READ, n=172), sarcoma (SARC, n=261), skin cutaneous melanoma (SKCM, n=470), stomach adenocarcinoma (STAD, n=443), testicular germ cell tumors (TGCT, n=150), thyroid carcinoma (THCA, n=507), uterine corpus endometrial carcinoma (UCEC, n=560), uterine carcinosarcoma (UCS, n=57), and uveal melanoma (UVM, n=80). For this analysis in each cancer, the LCK-high expressing population (the top 10%) was compared to the LCK-low population (bottom 10% in expression). This was compared to CYT which has been previously defined [14]. Briefly, total raw read counts per gene were converted to transcripts per million (TPM), which was calculated by dividing by the gene's maximum transcript length to provide a coverage depth estimate and scaling to sum to a total depth of 1e6 per sample. CYT was calculated as the as the geometric mean of

GZMA and PRF1 expression values in TPM, where similar high (top 10%) and low (bottom 10%) groups were compared.

Immunohistochemistry (IHC)

LCK protein expression was performed using immunohistochemistry on an independent cohort of 72 ovarian cancer samples using a commercially available anti-LCK antibody (HPA003494, Sigma-Aldrich). Additionally, CD8 and CD20 immunohistochemistry staining was performed in this cohort (CD20:SAB5600082, Sigma-Aldrich, CD8: CD8-4B11-L-CE, Leica Biosystems), and demographics and survival data was abstracted. All tumor tissue samples were collected under a protocol approved by MD Anderson Cancer Center Institutional Review Board. They were resected from the primary tumor site of previously untreated HGSOC patients with stage 3 and 4 diseases. A semi-quantitative IHC score was assigned by pathology collaborators including a senior gynecologic pathologist (C.P., M.S.), and as LCK status of the sample was not previously tested both pathologists were inherently blinded. For scoring purposes tissue LCK+ lymphocytes staining was as none (0, average of one or less LCK+ lymphocyte), low (1, less than 10 LCK+ lymphocytes), medium (2, greater than 10 but less than 40 LCK+ lymphocytes), and high (3, greater than 40 LCK+ lymphocytes or multiple germinal centers). The same cut offs were used for CD8 and CD20 positivity, and the counts were averaged over 3 fields for independent pathology samples.

IHC was additionally performed across a range of benign and malignant serous neoplasms on a tissue microarray (TMA), where counts were averaged over the 3 cores. All tissue was

obtained under an IRB approved protocol at the University of Virginia, and the TMA contained a spectrum of serous gynecological tissues, including normal fallopian tube epithelium obtained at the time of salpingo-oophorectomy for benign ovarian cystadenomas and high grade serous carcinomas. A total of 20 normal fallopian tube samples, 14 high grade ovarian serous carcinoma tissues, and 13 benign serous cystadenomas were compared. Each tissue specimen was represented as 3 independent cores on the TMA.

Statistical analysis

Descriptive statistics (n, percent, mean, standard deviation) were calculated to summarize patient demographics. Cox regression and backwards stepwise regressions were performed to assess OS and PFS for immune-related genes and dichotomized CYT groups. Statistical analyses were performed using SAS 9.4 for Windows (SAS Institute Inc., Cary, NC). IHC score comparison was performed using the Mann-Whitney U test with $p<0.05$ considered significant. Spearman correlations assessed the strength of association of LCK, CD20, and CD8. Strength of correlations analysis was performed using R version 3.4.1 package "cocor" [21].

Results

High LCK expression predicts improved survival in HGSOC

A total of 535 high grade serous ovarian samples in the TCGA dataset were included using the cBioPortal platform, 520 of which had Affymetrix U133 microarray data available for mRNA analysis [18]–[20]. Analysis of the TCGA was performed investigating the

upregulation of immune related genes including CD3E, CD3D, CD2, CD4, Perforin 1 (PRF1), Granzyme A (GZMA), CD19, and CD20 (MS4A1) and LCK (**Figure 1**). Of note, CD8A data were unavailable within the TCGA microarray dataset. High LCK mRNA expression was present in 23 (4%) of all cases (**Figure 1**). Progression free and overall survival data were collected for each of the above genes and compared in elevated and non-elevated samples. LCK was shown to have the strongest association with survival; patients with high LCK mRNA expression had a median progression free survival of 29.4 months, compared to 16.9 in those without high LCK expression (p=0.003**)**. Similarly, patients with high LCK had significantly longer overall survival than non-LCK high with median overall survival time of 95.1 months and 44.5 months respectively (p= 0.001). As expected, LCK mRNA high samples also had significantly higher LCK protein levels as determined by reverse phase protein arrays (RPPA). Only two other markers within the panel were statistically significantly associated with survival and were shown to have less dramatic prognostic differences. Specifically, high expression of B-cell marker CD20 (MS4A1) was associated with survival, with median PFS of 27.2 months (p= 0.08) and overall survival of 86.1 months (p=0.02), while CD3E elevation had a significant association with PFS (p=0.016) but was not associated with OS (p=0.330). High expression of the other immune related genes tested above was not associated with survival.

To examine if high LCK expression was simply a marker of high levels of tumor infiltrating lymphocytes (TIL), we compared the levels of CD3 and TCR related transcripts in LCK high samples. We also evaluated potential demographic, clinical, and pathological differences

between LCK high and remaining samples (**Table 1)**. The median age in the entire cohort was 59 years old (30-89 years), and most patients were advanced stage (72.9 % stage IIIC, 16.0% stage IV). No differences were detected between the two groups with respect to clinical characteristics, including age, race, ECOG performance status, clinical stage, and tumor grade. LCK expression was correlated with high expression of CD3 and TCR related transcripts, but as described above LCK had improved discriminatory prognostic ability than these markers alone.

Given the dramatic improvement in survival demonstrated in LCK-high samples, the influence of other established prognostic factors was tested in a Cox multivariable model that included LCK status, age, race (white vs other), stage, grade, and ECOG status. LCK status ($p=0.021$, HR=0.508) and race ($p=0.024$, HR = 0.657) were independent predictors of survival, ie reduced the risk of progression event. Additionally, LCK mRNA level improved OS ($p=.001$; HR=.315), as did race ($p=.038$; HR=.676) while age ($p<.001$; HR=1.026) increased the risk of death event.

High LCK does not correlate to increased mutation number

Non-synonymous somatic mutations in malignancies can lead to expression of "neo-epitopes" and hence increased potential immunogenicity, thus the relationship between LCK levels and number of somatic mutations in high grade serous ovarian cancer samples was evaluated. High mutation load, as defined by mutation count > 100, was present in 18 out of 520 tumors with sequencing data available (3.5%). To determine a possible relationship

between mutational load and LCK expression, the number of somatic mutations in LCK high samples was compared to that of non-LCK high tumors. This revealed no significant difference in mutation load or copy number alteration based on LCK expression status (**Figure 1**). In fact, in the LCK high samples, there was only one tumor with a mutation count greater than 100 (4.3% of the LCK high group).

LCK is a more significant prognostic predictor than CYT in ovarian cancer and many other malignancies

For this analysis, the definition of LCK high samples was liberalized (top 10%) and survival was compared to low LCK (bottom 10%) within the TCGA in order to reduce selection bias due to small numbers of LCK high/low cases. The median OS in the LCK high group was 52.6 months, as compared to 35.3 months in the LCK low group (p=0.00898). Similar dichotomization of CYT, a measure of transcript levels of perforin (PRF1) and granzyme A (GZMA), was performed; samples were grouped by CYT score into highest and lowest 10%. CYT did not predict survival, with median OS was 49.4 and 52.8 months in high and low cohorts respectively (p = 0.664). Kaplan-Meier curves can be found in **Figure 2**.

This analysis was performed for 30 tumor types available in TCGA (**Table 2**). Of these 30 cancer types, CYT was a significant predictor of overall survival in 5 cancers including: breast invasive carcinoma (BRCA, p=0.00293), cervical carcinoma (CESC, p = 0.0121), low grade glioma (LGG, p = 0.0112), sarcoma (SARC, p = 0.0323), and cutaneous melanoma (SKCM, p = 0.00509). The LCK high group also had statistically significant improved

survival in these subtypes (BRCA p=0.0546, CESC p= 0.000748, LGG p = 0.0269, SARC p = 0.0166, and SKCM p =0.0271). Interestingly, high LCK expression also had improved overall survival in an additional 3 cancer subtypes, namely: ovary as described above, head and neck squamous carcinoma (HNSC, p = 0.0496), and uterine carcinosarcoma (UCS, p = 0.0358). Therefore, LCK was a more discerning predictor in tumor types where CYT was predictive of OS, and it was additionally prognostic in a further subset of tumor types where CYT was not.

LCK protein expression independently confirms impact on prognosis

In order to determine if there was concordance between high LCK mRNA and protein expression we investigated LCK protein levels in samples designated as LCK-high by mRNA expression in the TCGA cohort using reverse phase protein arrays (RPPA). As expected, the LCK-high mRNA samples also expressed significantly higher levels of LCK protein. We also used an independent validation cohort of 72 high grade serous ovarian cancer samples with available clinical data to compare LCK protein expression using IHC with CD8, and CD20 (markers of cytotoxic Tlymphocytes and B-cells, respectively). This analysis confirmed that LCK expression was specific to tissue lymphocytes and that there was no confounding LCK expression by normal epithelial or by tumor cells. Furthermore, survival analysis revealed that only high LCK staining significantly increased overall survival, with median survival for high LCK staining of 40.5 months compared to 27.0 months (p=0.04, **Figure 3**). Neither LCK intensity nor LCK distribution (focal or diffuse) resulted in further stratification of the impact of LCK on survival.

Transcriptional profile differs in LCK high samples

Given the prognostic importance of high LCK expression, we used the availability the U133 microarray data as part of the TCGA dataset to evaluate gene expression differences between LCK-high expressing (n=23) and remaining samples (n=496). This analysis revealed 291 differentially expressed transcripts (at a statistical cut-off of $P<0.001$ and at least twofold change). As expected, LCK-high samples were characterized by higher expression of many transcripts associated with T cell function (**Appendix A**). For example, CD2, CD3, TRBC1, GZMA, GZMB, TRAC, and several HLA class I and II transcripts were all significantly higher expressed in LCK high samples. The greatest fold change was observed for Chemokine (CXC motif) ligand 9 (CXCL9, also known as chemokine induced by interferon γ (MIG)) with 15.64 higher expression level in the LCK high samples. Given that LCK is a canonical T lymphocyte signaling molecule, it was surprising to find that many B lymphocyte/plasma cell related transcripts including many immunoglobulin genes (e.g. IGHD, IGHM, IGKC, IGLJ3, IGLC1, and IGLV1-44) were also enriched in the LCK-high samples (**Appendix A)**. Interestingly, CXCL13 (also known as B lymphocyte chemoattractant (BLC)) was one of the chemokines enriched in LCK high samples (7.7 fold).

We next performed gene ontology enrichment analysis (**Table 3**), where genes are defined into subsets based on functional characteristics allowing for the biologic profile of the gene set to be obtained. This analysis confirmed that LCK high samples were significantly enriched in B cell function and activity, as demonstrated by the highest observed-to-expected

ratios in the "immunoglobulin complex circulating" gene ontology term (enrichment score: 46.41). In terms of molecular function, MHC II receptor activity was most closely correlated with an enrichment score of 41.73, followed by C-C chemokine binding (29.8), and this was mirrored in the biologic process analysis where MHC class II protein complex assembly had the greatest enrichment (32.44, **Table 3**).

Given the enrichment of B-cell transcripts in LCK high samples, we also investigated the presence of tertiary lymphoid structures (TLS) in the independent cohort of 72 HGSOC samples. TLS represent transient colocalization of lymphoid cells in non-lymphoid tissues; the presence of TLS has been described in multiple solid tumor types and is felt to influence local and potentially systemic anti-cancer response. We found that LCK expression by IHC was moderately correlated with TLS (Spearman correlation: 0.53, p= <0.0001). Proportional hazards regression analysis was performed including both TLS and LCK as predictors of OS, and both were significant independent predictors of survival (HR_{TLS} = 4.1, p=0.004, HR_{LCK}= 3.8, p=0.005). Finally, consistent with our mRNA expression analysis, there was moderate correlation between LCK, CD20, CD8 staining, but there was no evidence of any difference in strength of correlation between pairs of these markers (95% CI -0.18-0.28 for LCK/CD8 vs LCK/CD20 and 0.14-0.31 for CD20/CD8 vs LCK/CD8).

Given the prognostic significance of LCK positive lymphocytes in HGSOC, we next sought to determine if the abundance of such lymphocytes differed between normal fallopian tube epithelium (tissue of origin for the vast majority of HGSOC), benign serous neoplasms, and

HGSOC. LCK expression was evaluated by IHC in a TMA consisting of 20 normal fallopian tube samples, 13 serous cystadenomas, and 14 HGSOC samples. We observed higher LCK expression in the malignant samples than in their benign counterparts (p=0.023, **Appendix B)**. However, LCK expressing lymphocytes were present (albeit at lower prevalence) among normal fallopian tube epithelium samples, suggesting a possible surveillance or a tissue resident function.

Discussion

The immunogenicity of EOC has been well documented, with extensive literature demonstrating the presence of tumor infiltrating lymphocytes in ovarian tumors and their prognostic significance [2]–[8]. However, the biological basis and the identification of reliable markers for this prognostic significance have proven elusive. The original publication of the ovarian cancer TCGA analysis identified an "immunoreactive" group as one of the four subtypes of high grade serous ovarian cancer based on transcriptional profiling. However, there was no prognostic impact on survival associated with this immunoreactive subtype [18]. Recent publications have reported a histotype-specific nature of immune infiltration and have demonstrated that the magnitude of survival benefit in ovarian cancer was dose dependent on CD8 positive TILs [22], [23]. However, the use of TIL for clinical decision making currently remains in its early stages, and investigation into genomic markers have yielded mixed results.

The need for a robust, reproducible, and immune-related biomarker in HGSOC is further highlighted by the emerging data on immune checkpoint blockers resulting in response rates of 10-15% in heavily pretreated patients [14], [24]–[27]. Given the low response rates and significant toxicities of such therapies, studies aimed at identifying factors to provide more personalized prognostication for immune response in particular are of utmost importance. The use of PDL1 staining has emerged as a convenient and intuitive marker for prediction of response to immune checkpoint inhibitors, at least in some cancers. However, the predictive accuracy of this marker for ovarian cancer remains unknown. It is worth mentioning that the response rates to PD1/PDL1 targeting monoclonal antibodies is not appreciably higher in clinical trials that used PDL1 positivity by IHC as an eligibility criterion [26].

The current study demonstrates high LCK expression identifies a small subset of high grade serous ovarian cancers with better PFS and OS following treatment with standard frontline platinum-taxane adjuvant chemotherapy. LCK is an attractive biomarker as it plays a central functional role in T-cell signaling. The T-cell receptor (TCR) is composed of an antigen recognition subunit (TCRαβ) as well as three signaling subunits (CD3) [28]. TCR-CD3 engagement with antigen induces phosphorylation by LCK, which then triggers downstream signaling cascades leading to antigen specific T-cell immune response. Additionally, mice lacking LCK develop profound T cell deficiency [29]. Therefore, LCK is central to effective and specific T-cell response, including to tumor antigen. However, LCK is demonstrated herein to have greater discriminatory prognostic ability than previously validated metrics of

T cell function such as CYT, which suggests it may capture additional facets of tumoral immune response such as B cell activity.

The impact of B cell infiltrates in ovarian malignancy is less clear than their T-cell counterparts, though they have been shown to similarly be associated with improved survival [12], [13], [30]. The role of B cells has been supported by prior analysis of the TCGA, which demonstrated improved survival with B-cell gene expression signatures in high grade serous ovarian cancer [31]. The causality and mechanism of the herein reported correlation between LCK and B cell signatures remains to be determined. Prior literature suggests B cells may induce the maturation of dendritic cells making them competent for T-cell activation, and preclinical studies demonstrate depletion of B cells in a mouse model results in decreased expression of the degranulation marker CD107 on CD8+ T cells, suggesting impaired cytotoxic response [32], [33]. Interestingly, LCK has also been implicated in B-cell signaling at least in a minor but important B-cell subset, namely B-1 cells. These cells are found predominantly in peritoneal and pleural cavities, which are notably the primary location of ovarian cancer spread, and are characterized by deficient B-cell receptor (BCR) signaling [30], [31]. In future studies we plan to further investigate the potential prognostic significance of B1-cells and their LCK expression in HGSOC.

The limitations of the current research include small sample size, specifically due to the stringent criteria of top 3%; the low number of LCK high tumors within the TCGA limits the power of this analysis, specifically for gene enrichment and ontology. However, for all

subsequent analyses, more liberal definitions of LCK high tumors were used, including top 10% for comparison with CYT and pathologic criteria for IHC in the independent cohort. Therefore, the consistency of the association between LCK and survival lends strength to this conclusion. For the comparison to CYT, the high and low cohorts were defined arbitrarily, as has been done in other analyses; for example, significance of CYT in pancreas defined top decile and compared to bottom quartile resulting in a difference in significance level [17].

In summary, this study demonstrates high LCK expression is associated with significantly longer survival than non-high LCK tumors, and was found to be a more significant predictor of prognosis than the previously validated cytolytic activity score (CYT) across tumor types, including HGSOC. LCK high samples demonstrated evidence of enriched B cell infiltration and function raising the possibility a cooperative interaction between tumor infiltrating T and B cells is correlated with better survival in this disease. Further research is needed to better elucidate the causality and mechanism of this correlation.

CONCLUSION

In this study, we sought to establish genomic biomarkers in ovarian malignancy which capture the known immunogenicity of this tumor type and its relationship to prognosis. We utilized the Cancer Genome Atlas (TCGA) to demonstrate high LCK mRNA expression was a strong predictor of survival in a set of 535 ovarian cancers. Patients with high LCK mRNA expression had a longer median progression free survival (PFS) and overall survival (OS). We then confirmed this association of LCK with survival in an independent cohort, and importantly used less stringent cut offs for definition of high expressing tumors which allows our findings to be generalized to a greater subset of ovarian malignancy. Additionally, LCK expression was compared to a previously validated metric, cytolytic activity score (CYT), in order to determine their respective prognostic capacity. Across tumor types available in the TCGA, LCK was a more significant predictor of prognosis; LCK was a more discerning predictor in tumor types where CYT was predictive of OS, and it was additionally prognostic in a further subset of tumor types where CYT was not in HGSOC.

In an attempt to generate alternate hypotheses about the mechanism for the improved prognostic ability of LCK, we used the ovarian cancer TCGA dataset to evaluate gene expression differences between LCK-high expressing and the remaining samples. Given that LCK is a canonical T lymphocyte signaling molecule, it was surprising to find many B lymphocyte/plasma cell related transcripts including many immunoglobulin genes were also

enriched in the LCK-high samples. This research suggests a cooperative interaction between tumor infiltrating T and B cells may correlate with better survival in this disease, and this relationship is captured by LCK expression and is not reflected by other metrics which are specifically T-cell focused such as CYT.

To date, most studies evaluating the prognostic significance of TILs have concentrated on T cells, while less attention has been devoted toward TIL-B cells. In ovarian cancer there is conflicting evidence on the association between B-cells and survival. TIL-B cells may function to present tumor antigen to cytotoxic T cells or other immune effector cells, and plasma cells may secrete antibodies aiding the immune response against tumor cells. Alternatively, TIL-B subsets may function to suppress T cell anti-tumor responses (as in Bregs) or promote tumor progression by nurturing an inflammatory microenvironment. Therefore, it will be important to build upon the data presented herein to investigate these B-cell signatures within the TCGA. Additionally, in our independent cohort, further investigation of tertiary lymphoid structures, which may serve as an immunohistochemical and pathologic marker of T and B cell cooperation, should be reviewed.

The limitations of the current research include small sample size, specifically due to the stringent criteria of top 3%; the low number of LCK high tumors within the TCGA limits the power of this analysis, specifically for gene enrichment and ontology. However, for all subsequent analyses, more liberal definitions of LCK high tumors were used, including top 10% for comparison with CYT and pathologic criteria for IHC in the independent cohort.

Therefore, the consistency of the association between LCK and survival lends strength to this conclusion. For the comparison to CYT, the high and low cohorts were defined arbitrarily, however this is similar to the approach in other analyses.

In summary, high LCK expression is a better prognostic marker than the previously validated cytolytic activity score (CYT) across tumor types, which we argue is due to its ability to capture T and B cell cooperation, given that LCK high samples demonstrated evidence of enriched B cell infiltration and function. Further research is needed to better elucidate the causality and mechanism of this correlation. Improved understanding of these relationships may offer valuable therapeutic approaches for the treatment of ovarian cancer, particularly in patients with drug or immune therapy resistant disease.

TABLES

Table 1: Demographics by LCK Expression Level

Total Cohort† 520	LCK High* n=23	Non-LCK High n=497	
Characteristic			p-value
Age (median)	40-78 (58)	30-89 (59)	0.837
ECOG Performance			
0	4	69	
1	3	72	0.633
2	2	21	
3	0	4	
Unknown	14	331	
Stage			
I	1	15	
II	3	25	
IIIA,B	3	28	0.134
IIIC	13	366	
IV	3	80	
Unknown	0	4	
Grade			
1	0	5	
2	3	61	0.552
3	19	419	
Unknown	1	12	
Race/Ethnicity			
Asian	1	14	
Black	0	23	0.4696
Hispanic	1	7	
White	20	433	
Other/Unknown	1	20	

†520 patients included from a total of 535 samples available

*LCK (lymphocyte specific tyrosine kinase) high: expression >1.86SD within TCGA ovarian serous cystadenocarcinoma study (TCGA, provisional).

Table 2: Survival Analyses Comparing the Prognostic Ability of LCK and CYT

Cancer subtype[1]	LCK			Cytolytic Activity Score (CYT)		
	Median OS bottom 10% (months)	Median OS top 10% (months)	P value	Median OS bottom 10% (months)	Median OS top 10% (months)	P value
ACC	NA	NA	0.818	NA	NA	0.990
BLCA	NA	94.3	0.254	NA	NA	0.506
BRCA	90.4	132	0.055	84.5	NA	0.003
CESC	19.4	NA	0.001	136	NA	0.012
CHOL	24.7	NA	0.870	9.03	NA	0.642
COAD	NA	NA	0.363	NA	NA	0.863
ESCA	42.1	26.1	0.930	26.1	16.1	0.617
GBM	13.2	12.5	0.623	13.2	10.6	0.295
HNSC	85.7	161.9	0.050	28.7	58.7	0.109
KIRC	NA	66	0.497	NA	73	0.473
KIRP	NA	NA	0.232	NA	98	0.591
LAML	12.2	10.1	0.118	26.4	10.2	0.084
LGG	63	63.8	0.027	81.1	52.6	0.011
LIHC	NA	54.1	0.865	59.7	56.2	0.763
LUAD	48.5	87.2	0.368	49.7	43.1	0.664
LUSC	74.1	56	0.603	74.1	61.9	0.918
MESO	17.6	13.8	0.584	25.2	13.8	0.959
OV	35.3	52.6	0.009	52.8	49.4	0.664
PAAD	NA	23.4	0.687	21.7	50.1	0.973
PCPG	NA	NA	0.429	NA	NA	0.317
PRAD	NA	NA	0.304	NA	NA	0.893
READ	NA	NA	0.317	NA	NA	0.221
SARC	35.4	NA	0.017	41.2	NA	0.032
SKCM	54.3	164.3	0.027	58.9	164.3	0.005
STAD	58.2	22.3	0.857	73.2	NA	0.936
TGCT	NA	NA	0.317	NA	NA	0.289
THCA	NA	NA	0.631	NA	NA	0.659
UCEC	NA	NA	0.221	NA	NA	0.263
UCS	22.8	30.4	0.036	31.6	NA	0.804
UVM	NA	NA	0.808	NA	NA	0.806

Median overall survival in high LCK expression and low LCK expression as compared to high and low CYT score. High and low groups are defined as top 10% and bottom 10% respectively.

[1] The following tumor types (project code and n=sample size) were included: adrenocortical carcinoma (ACC, n=92), bladder/urothelial (BLCA, n=412), breast invasive carcinoma (BRCA, n=1098), cervical squamous cell carcinoma and endocervical adenocarcinoma (CESC, n=307), cholangiocarcinoma (CHOL, n=51), colon adenocarcinoma (COAD, n=461), esophageal carcinoma (ESCA, n = 185), glioblastoma multiforme (GBM,

n=617), head and neck squamous cell carcinoma (HNSC, n=528), kidney renal clear cell carcinoma (KIRC, n=537), kidney renal papillary cell carcinoma (KIRP, n=291), acute myeloid leukemia (LAML, n=200), low grade glioma (LGG, n=516), liver hepatocellular carcinoma (LIHC, n=377), lung adenocarcinoma (LUAD, n = 585), lung squamous cell carcinoma (LUSC, n=504), mesothelioma (MESO, n=87), ovarian serous cystadenocarcinoma (OV, n=608), pancreatic adenocarcinoma (PAAD, n=185), pheochromocytoma and paraganglioma (PCPG, n=179), prostate adenocarcinoma (PRAD, n=500), rectum adenocarcinoma (READ, n=172), sarcoma (SARC, n=261), skin cutaneous melanoma (SKCM, n=470), stomach adenocarcinoma (STAD, n=443), testicular germ cell tumors (TGCT, n=150), thyroid carcinoma (THCA, n=507), uterine corpus endometrial carcinoma (UCEC, n=560), uterine carcinosarcoma (UCS, n=57), and uveal melanoma (UVM, n=80).

Table 3: Gene Ontology Enrichment in selected subset (LCK high)

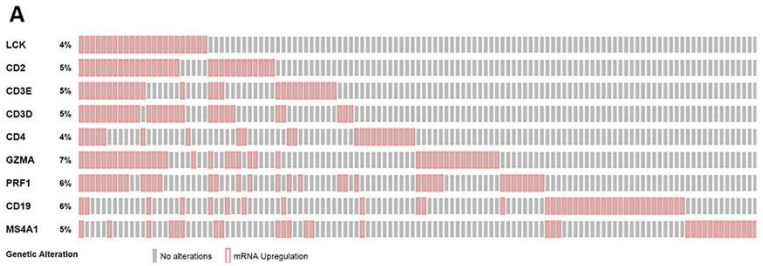
Cellular Component

GO ID	GO Term	Observed in selected subset	Expected in selected subset	Observed/ Expected*
GO:0042571	immunoglobulin complex, circulating	7	0.15	46.41
GO:0019814	immunoglobulin complex	7	0.22	32.48
GO:0042612	MHC class I protein complex	6	0.26	23.2
GO:0061702	inflammasome complex	6	0.3	19.89
GO:0042101	T cell receptor complex	6	0.39	15.47
Molecular Function				
GO:0032395	MHC class II receptor activity	7	0.17	41.73
GO:0019957	C-C chemokine binding	5	0.17	29.8
GO:0046977	TAP binding	6	0.22	26.82
GO:0019865	immunoglobulin binding	6	0.24	24.76
GO:0004950	chemokine receptor activity	8	0.34	23.84
GO:0001637	G-protein coupled chemoattractant receptor activity	8	0.34	23.84
GO:0023026	MHC class II protein complex binding	6	0.26	22.99
GO:0019956	chemokine binding	6	0.28	21.46
GO:0045236	CXCR chemokine receptor binding	6	0.3	20.12
GO:0023023	MHC protein complex binding	6	0.3	20.12
Biological Process				
GO:0002399	MHC class II protein complex assembly	5	0.15	32.44
GO:0046113	nucleobase catabolic process	5	0.18	27.8
GO:0002396	MHC protein complex assembly	5	0.18	27.8
GO:0010818	T cell chemotaxis	9	0.39	23.35
GO:0002480	antigen processing and presentation of exogenous peptide antigen via MHC class I, TAP-independent	5	0.23	21.62
GO:0090026	positive regulation of monocyte chemotaxis	7	0.36	19.46
GO:0010819	regulation of T cell chemotaxis	5	0.26	19.46
GO:1901623	regulation of lymphocyte chemotaxis	9	0.49	18.44
GO:0036037	CD8-positive, alpha-beta T cell activation	5	0.31	16.22

* Observed/Expected <15.0 not reported

FIGURES

Figure 1: TCGA Analysis of Immune-related Gene Expression



B

Gene (EXP>1.86)	PFS	p-value	OS	p-value
LCK	29.4	0.003	95.1	0.001
CD2	24.41	0.117	58.6	0.096
CD3E	27.7	0.016	58.1	0.33
CD3D	24.41	0.066	85	0.056
CD4	16.8	0.422	44.5	0.418
GZMA	22.3	0.314	58.6	0.647
PRF1	15.5	0.978	44.9	0.771
CD19	27.8	0.147	63	0.148
MS4A1	27.2	0.083	86.1	0.021

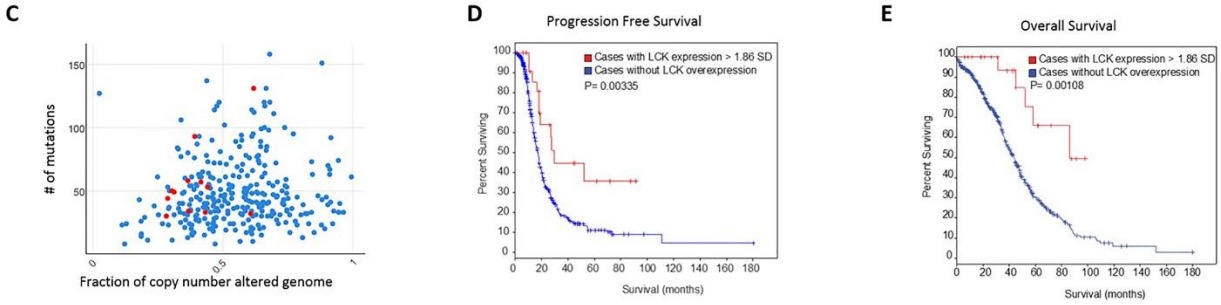


Figure 1: TCGA Analysis of Immune-related Gene Expression

(A) Altered gene expression samples: total mutations per sample at top, expression levels (>1.86 SD) of common immune related genes by sample. (B) Kaplan Meier analysis of progression free survival and overall survival in gene-high as compared to not gene-high samples for respective immune related genes. (C) Mutation count and copy number alterations among total study tumors (blue) and LCK high tumors (red). (D) Kaplan Meier analysis of progression free survival in LCK high (red) tumors compared to non-LCK high (blue). (E) Kaplan Meier analysis of overall survival in LCK high (red) tumors compared to non-LCK high (blue).

Figure 2: Kaplan Meier Analysis Comparing Prognostic Ability of LCK and CYT

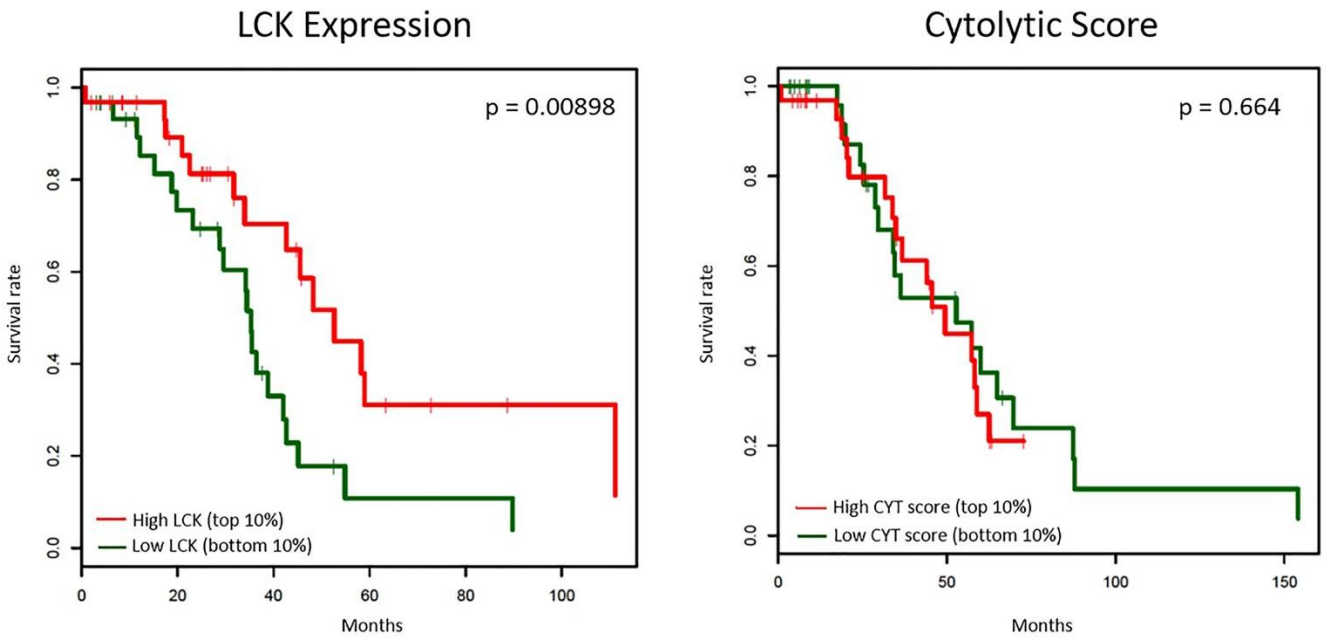


Figure 2: Kaplan Meier analysis comparing the prognostic ability of LCK and CYT

(A) Kaplan Meier analysis of overall survival in high LCK expression (top 10%, red) as compared to low LCK expression (bottom 10%, green).
(B) Kaplan Meier analysis of overall survival in high CYT score (top 10%, red) as compared to low CYT score (bottom 10%, green).

Figure 3: LCK Expression and Survival Analysis in an Independent Cohort

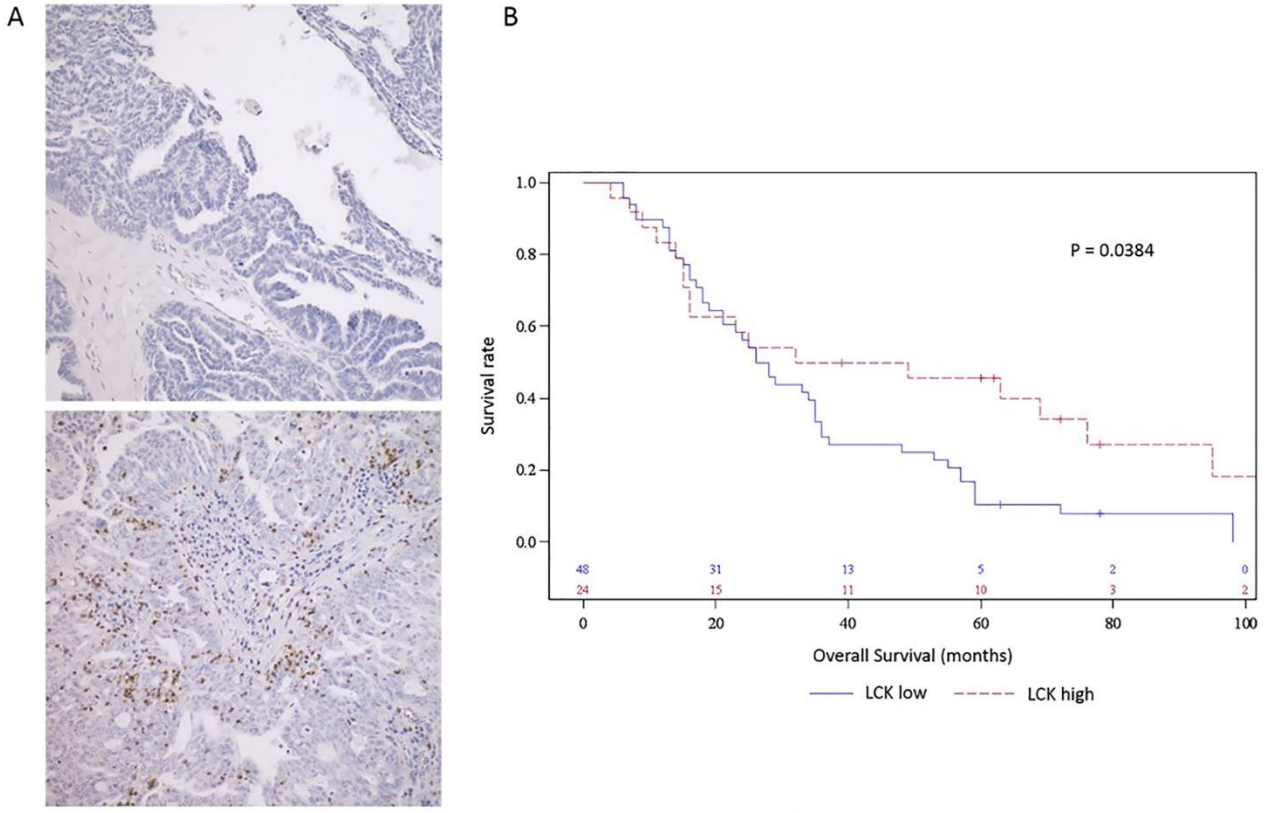


Figure 3: LCK expression and Survival Analysis in an Independent Cohort (A) Representative examples of varying LCK expression by immunohistochemistry. Top: LCK low expression in high grade serous ovarian cancer. Bottom: LCK high expression in high grade serous ovarian cancer. (B) Kaplan Meier analysis of overall survival in high LCK expression (red) as compared to low LCK expression (blue).

APPENDICES

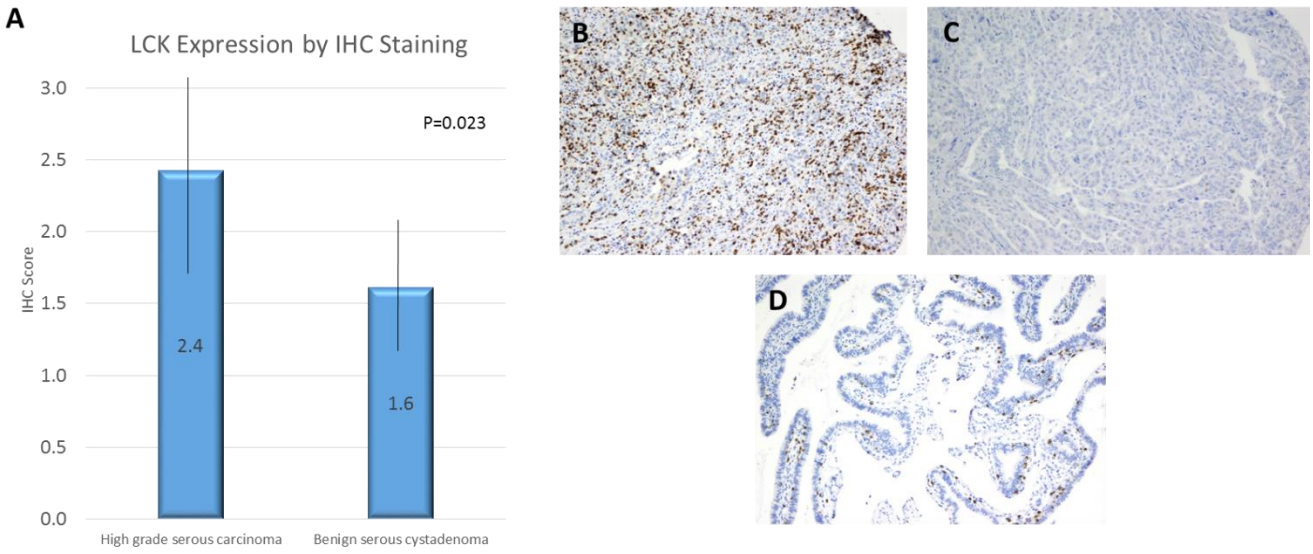
Appendix A: Top Overexpressed Genes in LCK High Samples

Symbol	Name	Geometric mean of intensities in class 1*	Geometric mean of intensities in class 2**	Fold change[ı]	Parametric p-value	FDR
CXCL9	chemokine (C-X-C motif) ligand 9	55.65	870.35	15.639712	< 1e-07	< 1e-07
IGLC1	immunoglobulin lambda constant 1 (Mcg marker)	186.45	1984.25	10.642263	< 1e-07	< 1e-07
IGHM	immunoglobulin heavy constant mu	26.53	221	8.3301922	< 1e-07	< 1e-07
IGKC	immunoglobulin kappa constant	26.11	226.8	8.6863271	< 1e-07	< 1e-07
JCHAIN	joining chain of multimeric IgA and IgM	25.76	217.56	8.4456522	< 1e-07	< 1e-07
IGKC	immunoglobulin kappa constant	27.05	221.33	8.1822551	< 1e-07	< 1e-07
CXCL13	chemokine (C-X-C motif) ligand 13	18.99	151.74	7.9905213	< 1e-07	< 1e-07
IGHM	immunoglobulin heavy constant mu	16.18	113.07	6.9882571	< 1e-07	< 1e-07
TRBC1	T cell receptor beta constant 1	32.03	208.74	6.5170153	< 1e-07	< 1e-07
IGLJ3	immunoglobulin lambda joining 3	23.3	154.25	6.6201717	< 1e-07	< 1e-07
IGKC	immunoglobulin kappa constant	103.1	681.32	6.6083414	< 1e-07	< 1e-07
CCL5	chemokine (C-C motif) ligand 5	27.65	175.54	6.3486438	< 1e-07	< 1e-07
TRBC1	T cell receptor beta constant 1	31.07	187.03	6.0196331	< 1e-07	< 1e-07
IGLC1	immunoglobulin lambda constant 1 (Mcg marker)	19.22	109.64	5.7044745	< 1e-07	< 1e-07
CD2	CD2 molecule	24.16	129.36	5.3543046	< 1e-07	< 1e-07
IGLJ3	immunoglobulin lambda joining 3	13.85	71.54	5.165343	< 1e-07	< 1e-07
CD8A	CD8a molecule	16.17	82.27	5.0878169	< 1e-07	< 1e-07
CD3D	CD3d molecule, delta (CD3-TCR complex)	34.53	175.11	5.0712424	< 1e-07	< 1e-07
IGLV1-44	immunoglobulin lambda variable 1-44	14.17	72.32	5.1037403	< 1e-07	< 1e-07

*Class1 = non-LCK high ** Class2 = LCK high

ı Fold change < 5.0 are not reported

Appendix B: LCK Expression in Benign and Malignant Tissue



(A) LCK expression levels by immunohistochemistry staining score. Staining score defined as: 0=none, 1=low, 2= medium, 3 = high. (B-D) Representative examples of varying LCK expression by immunohistochemistry. B: LCK high expression in high grade serous ovarian cancer. C: non LCK high expression (low) in high grade serous ovarian cancer. D: Moderate LCK expression in normal fallopian tube (medium).

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