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August 5, 2020

Albert Roessner

Executive Editor, *Pathology - Research and Practice*

Dear Dr. Roessner,

Please find enclosed our manuscript entitled *"Can Immune Markers Help Identify Fast Relapse in Patients with Muscle Invasive Bladder Cancer?"* which we are submitting for consideration of publication as a short communication in "*Pathology - Research and Practice*".

The aim of this pilot study was to assess the role of immune markers in predicting fast relapse (<2 years) in a cystectomy cohort (n=40) of urothelial carcinomas. In brief, we report strong correlation between low CD3, CD4 and CD8 expression and fast relapse. Additional evaluation of the immune transcriptome by NanoString Human PanCancer Immune Panel v.1.1 has identified 5 differentially expressed genes significantly associated with fast relapse. Among these, KLRB1 and HLA-DQA1 were also significant on Kaplan-Meier analysis. These findings strengthen the potential clinical utility and, hence, the need for further evaluation of immune markers in HGUC prognostication. We believe these findings will be of great interest to the readers of *Pathology - Research and Practice*.

All authors of this manuscript have directly participated in the planning, execution, or analysis of the study. All authors have read and approved the final version of the manuscript. The contents of the manuscript have not been copyrighted or published previously, nor are they under consideration for publication elsewhere. Furthermore, the contents of the manuscript will not be copyrighted, submitted or published elsewhere while acceptance by the journal is under consideration. Dr. Downes has been an advisory board member for Astra Zeneca and Hoffman La Roche and has received speaker's honoraria from Astra Zeneca. All other authors have no conflict of interest to declare.

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We look forward to your response to this manuscript. If you have any questions or concerns, please do not hesitate to contact me.

Yours Sincerely,

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Can Immune Markers Help Identify Fast Relapse in Patients With Muscle Invasive Bladder Cancer?

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Abstract

The aim of this pilot study was to assess the role of immune markers in fast relapse (<2 years) of high-grade muscle invasive urothelial carcinomas of the bladder (HGUC) treated by cystectomy. A series of 40 such cases was investigated for immune protein (CD3, CD4, CD8, CD20, CD68, CD163, FOXP3 and PD-1) status by immunohistochemistry. Decreased expression of all immune cell markers was observed in tumors of patients who relapsed quickly. In Kaplan-Meier (log-rank test) analysis, low CD3, CD4 and CD8 expression was associated with fast relapse (P= 0.005, 0.028, 0.036 respectively). Additional evaluation of the immune transcriptome by NanoString Human PanCancer Immune Panel v.1.1 has identified 5 differentially expressed genes significantly associated with fast relapse. Among these, *KLRB1* and *HLA-DQA1* were also significant on Kaplan-Meier analysis (log-rank test P=0.007 and 0.006, respectively). These findings strengthen the potential clinical utility and, hence, the need for further evaluation of immune markers in HGUC prognostication.

Keywords: Urothelial carcinoma, prognostication, inflammation, immune microenvironment, gene expression profiles, NanoString technology.

1. Introduction

Bladder cancer (BC) is the 9th most commonly diagnosed cancer and the 13th leading cause of cancer death worldwide [1]. At primary diagnosis, up-to 25% of patients present with muscle-invasive bladder cancer (MIBC). Additionally, among non-muscle invasive disease, progression to MIBC occurs in 20%-30% of patients. The current standard of care for MIBC consists of radical cystectomy (RC), with bilateral lymphadenectomy and platinum-based - chemotherapy in patients with advanced and metastatic disease [2]. However, approximately 50% of patients with high-grade urothelial carcinoma (HGUC) experience relapse and/or death, with the majority (>80%) occurring within the first 2 years post-surgery [3].

Prognostic factors for HGUC recurrence and death post-RC include advanced disease stage, grade and nodal involvement [4]. Nomograms integrating lymphovascular invasion (LVI), status of soft tissue (ST) margins, patient age and gender have also been developed, but prognostication remains suboptimal [5]. In this regard, the tumor immune microenvironment is becoming increasingly relevant for prediction of response to therapy and disease prognostication. Owing to advances in immunotherapy and accelerated approvals by the US Food and Drug Administration (FDA) of programmed cell death protein (PD-1) and programmed death ligand 1 (PD-L1) inhibitors in the treatment of advanced and metastatic UC, a paradigm shift in the treatment of these patients has recently occurred [6, 7]. Yet, only a subset of HGUC patients derive clinical benefit. The most promising predictive biomarkers in this therapeutic setting are the expression of PD-L1 and high tumor mutational burden, but with limited success [8].

In this pilot study, we performed NanoString-based immune transcriptome and immunohistochemistry-based immune protein profiling to gain insight into the tumor immune

microenvironment of HGUC patients. Prognostically relevant immune markers were identified by comparing patients who relapsed and/or died within 2 years post-RC versus those that did not.

2. Materials and methods

2.1 Case selection and review

This study was approved by the Research Ethics Board at Sunnybrook Health Sciences Centre (REB 187-2016). A subset of 40 HGUC (≥pT2) RC cases treated 1999-2015 was identified from our larger cohort of 235 MIBC cases through a retrospective search of the laboratory information system, Sunquest CoPath, as previously described [9]. Exclusion criteria were non-urothelial histology, presence of any neuroendocrine carcinoma component, and divergent differentiation encompassing >50% of the tumor. Only tumors with clinical follow up and sufficient tumor blocks were selected for the NanoString work. The complete set of hematoxylin and eosin (H&E)-stained slides from each cystectomy was collected and reviewed by a pathologist with subspecialty training in genitourinary pathology (M.R.D.), to confirm the following: tumor histology, grade (according to the 2016 World Health Organization/International Society of Urological Pathology guidelines), pathological stage [American Joint Committee on Cancer Immunohistochemistry (AJCC)/TNM 8th edition], the presence of carcinoma in situ (CIS), lymphovascular invasion, margin status, and the presence of nodal metastases. The following demographic data were recorded for each case: age, sex, date of surgery, date of last known follow-up, date of disease relapse (if applicable), and date of death (if applicable).

2.2 Evaluation of invasive front inflammation

Evaluation of invasive front inflammation was performed as previously described [9] . The slides containing UC were selected and inflammation was semi-quantitatively graded by two pathologists (A.H. and M.R.D.) with the four-point system developed by Klintrup and Makinen

[10]. In brief, inflammation was evaluated at the invasive front (defined as the deepest interface of carcinoma with stroma) and assigned a score of 0, 1, 2 or 3 (0, no inflammation; 1, patchy inflammatory infiltrate; 2, band- like infiltrate; and 3, prominent inflammation with destruction of UC cells). The scores were subsequently dichotomized as low (score 0 or 1) and high (score 2 or 3), as in the original publication [10].

2.3 Tissue Microarray (TMA) construction

Triplicate-core TMAs were created as previously described [9]. In brief, three independent sites containing tumour were circled on a single representative slide from each case. Triplicate 1-mm- core TMAs were constructed with a TMA instrument (Beecher Instruments, Silver Springs, MD, USA) used to punch the areas of interest from the respective tumour blocks. Four-micrometre- thick unstained slides were prepared from the TMA block for subsequent H&E and immunohistochemical staining, as described below.

2.4 Immunohistochemistry

Serial 4μm thick unstained TMA sections were cut and stained for CD3, CD4, CD8, CD20, CD68, CD163 and PD-1, as previously described [9]. Immunostained slides were evaluated for membranous staining in lymphocytes. Additionally, FOXP3 staining was performed and nuclear lymphocytic expression of FOXP3 was scored. All immune infiltrate staining was assessed as absolute hotspot counts (one representative x40 field/core), with the results being averaged across all cores for each case. Interpretation of immunohistochemically stained slides was conducted by one genitourinary pathologist (M.R.D.) blinded to all case characteristics.

2.5 RNA isolation and gene expression profiling

For each patient in the cohort, a single representative slide of the tumor was selected for RNA extraction as previously described [11]. Formalin-fixed, paraffin-embedded (FFPE) tissue

blocks matching the selected H&E slides were sectioned at a thickness of 5 μm (4-10 sections per case). These tissue slides were then superimposed on the H&E slides and each representative area of tissue was outlined. The circled area of tissue was then scraped with a scalpel and placed into 1.5 mL tubes. Total RNA was extracted from macrodissected FFPE tissue sections using the High-Pure FFPET RNA Isolation Kit (Roche Diagnostics, Indianapolis, IN, USA), per manufacturer's instructions. RNA was dissolved in nuclease-free water and quantified using a NanoDrop-1000 (Thermo Fisher Scientific, Waltham, MA, USA). RNA profiling was performed with 250ng of RNA and using the NanoString nCounter® Human v.1.1 PanCancer Immune Profiling Panel (NanoString Technologies Inc., Seattle, Washington, USA), according to the manufacturer's instructions.

Raw gene expression data was analyzed using NanoString's software nSolver v4.0 with the Advanced Analysis 2.0 plugin. Data normalization was performed on background-subtracted samples using internal positive controls and 36 selected housekeeping genes that were identified using the nSolver normalization module, which utilizes the geNorm algorithm (https://genorm.cmgg.be/). Differential gene expression analyses were performed using nSolver, which employs several multivariate linear regression models to identify significant genes (mixture of negative binomial, simplified negative binomial, or log-linear model). Resulting *P*-values were adjusted using the Benjamini-Hochberg (BH) method to control the false discovery rate (FDR). Statistically significant, differentially expressed genes (DEGs) were defined as those with expression levels corresponding to a log2 ratio >1 or < −1 and BH *q* value < 0.05.

2.6 Statistical Analysis

All statistical analyses were performed using SPSS 24.0 (IBM Corporation, New York, NY, USA). The Mann Whitney U-test was used to compare immune markers between patients

who relapsed quickly (defined as relapse and/or death within 2 years) post-RC vs. those that did not. Kaplan-Meier survival analysis and log-rank tests were utilized to assess the prognostic significance of clinicopathological characteristics and immune markers. Multivariate analysis was not undertaken to prevent overfitting of data.

3. Results

3.1 Clinicopathological predictors of fast relapse post radical cystectomy

The mean patient age was 70 years (range, 33-88 years) and the male/female ratio was 2.3:1. All were MIBC. Half the cohort (n=20, 50%) had low invasive front inflammation and half had high invasive front inflammation. Fast relapse, defined as relapse and/or death within 2 years post-RC, occurred in 63% (25/40) patients, mean follow-up time 25.1 months (range, 0-206 months). Nearly equal number of fast relapses occurred in the node negative group (n=11) as in the node positive group (n=12). Clinicopathological characteristics of the cohort are shown in Table 1.

Kaplan-Meier survival analysis (log-rank test) showed significant association between Lymphovascular invasion (LVI, P= 0.019), ST margins (P= 0.032), lymph node status (P= 0.002), low invasive front inflammation (P= 0.004) and fast relapse. Carcinoma in situ (CIS) showed no significant correlation (P=0.194). Multivariate analysis was not undertaken to prevent overfitting of data.

3.2 Immune protein markers associated with HGUC fast relapse

To assess the composition of the tumor immune microenvironment, we produced a TMA and applied antibodies for CD3, CD4, CD8 and CD20 (T-cells), CD68 and CD163 (tumor-associated macrophages), FOXP3 (regulatory T-lymphocyte marker) and PD-1 immune checkpoint protein. We found lower levels of all immune cell markers in MIBC tumors of patients

who relapsed quickly post-RC, although statistical significance was reached only for T-cell markers (CD3, CD4, CD8 and CD20) and PD-1 (Mann Whitney *P*= 0.006, 0.024, 0.021, 0.015, 0.005, respectively). Further, we identified an association between CD3, CD4 and CD8 expression and time to relapse (*P*= 0.005, 0.028, 0.036 respectively, Figure 1).

3.3 Immune-related RNA panel predictive of fast relapse post cystectomy

To define immune-related gene expression signatures associated with HGUC fast relapse, we performed targeted gene expression profiling using a panel of 770 tumor- and immune-related genes on the NanoString nCounter platform (nCounter human PanCancer immune panel). We then investigated differences in immune gene expression patterns between patients who relapsed quickly post-RC vs. those that did not, utilizing linear regression analysis. We identified 5 significantly (fold change >2 or < −2 and *P*-value < 0.05) differentially expressed genes (DEG); *CCL21* and *CR2* were upregulated while *KLRB1, CD36* and *HLA-DQA1* were downregulated in HGUC fast relapse (Figure 2A).

On Kaplan-Meier survival analysis (Figure 2B-F), HGUC patients were divided into two groups based on median gene expression level and prognostic differences between patients with high (above median) vs. low (below median) expression were evaluated by log-rank test. This analysis has identified significant association between low expression *KLRB1* (lymphocyte receptor) and *HLA-DQA1* (member of the MHC class II proteins) and fast relapse post-RC.

4. Discussion

HGUC patients have drastically different clinical outcomes following surgical resection. Some achieve long-term disease free survival while others go on to relapse, with over 80% relapsing within 2 years (fast relapse), and a subset suffering relatively rapid death from disease. Our data confirms that the most prognostically relevant RC pathological parameters in the setting

of HGUC fast relapse are LVI, ST margins and lymph node status. However, these pathological determinants fail to account for tumor biology, altered molecular pathways and therapeutic targets for anticancer therapy selection.

In this pilot study, we assessed inflammation in association with HGUC fast relapse in MIBC tumors. Consistent with the literature, we found low invasive front inflammation to be a predictor of unfavourable outcome [12]. Kaplan-Meier survival (log-rank test) analysis showed that downregulation of T-cell markers (CD3, CD4, CD8 and CD20) and upregulation of PD-1 immune checkpoint protein are associated with fast relapse. These results are in line with prior findings and reflect the key role of T-cells and their mediators in active anti-tumor response [13]. Although infiltration of other immune cell subsets, including FOXP3 regulatory T-cells and tumor-associated macrophages, have been previously correlated with improved survival in UC, in our cohort, CD68, CD163 and FOXP3 markers were not significant, possibly due to the small cohort size.

Gene expression profiles have the potential to more comprehensively assess the immune milieu and tumor microenvironment of HGUC, thus we next performed a NanoString-based immune transcriptome analysis of our cohort. The most significant DEG associated with fast relapse was *HLA-DQA1*, a class II alpha chain paralogue of the human lymphocyte antigen (HLA). Class II HLA molecules play a central role in the immune system by presenting peptides derived from exogenously acquired protein antigens to class II-restricted CD4 T-cells and promote anti-tumor immune response [14]. The second DEG was *KLRB1*, encoding CD161, a surface marker on numerous T-cell lineages and natural killer cells. It is a marker of enhanced innate T-cell immunity, known to be suppressed in tumor tissues and a pan-cancer marker of poor outcome [15]. Taken together, decreased expression of *HLA-DQA1* and *KLRB1* in HGUC may lead to escape

from T-cell mediated immune surveillance and may promote disease relapse. Larger comprehensive RNA profiling studies from multiple institutions are needed to further elucidate the biological significance and clinical relevance of *HLA-DQA1* and *KLRB1* in HGUC relapse.

In conclusion, our pilot study demonstrates LVI, ST margins, lymph node status, low invasive front inflammation, decrease in T-cell markers (CD3, CD4, CD8 and CD20) and increased PD-1 immune checkpoint protein expression are associated with HGUC fast relapse post-RC. Additionally, low RNA expression of *KLRB1* and *HLA-DQA1* is significantly associated with fast relapse post-RC. Both genes have putative roles in tumor escape from T-cell mediated immune surveillance that warrants further investigation. The main limitations of this preliminary analysis are the small cohort size and use of retrospective material from a single institution. Nevertheless, these findings strengthen the potential clinical utility and, hence, the need for further evaluation of immune markers in HGUC prognostication.

Abbreviations

CIS	Carcinoma in situ
DEG	Differentially expressed genes
FDA	US Food and Drug Administration
HGUC	High-grade urothelial carcinoma
LVI	Lymphovascular invasion
MIBC	Muscle-invasive bladder cancer
PD-1	Programmed cell death protein
PD-L1	Programmed death ligand 1
RC	Radical cystectomy
ST	Soft tissue

TMA Tissue Microarray

Declaration

Ethics statement: This study was approved by the Research Ethics Board at Sunnybrook Health Sciences Centre (REB 187-2016).

Data availability: The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Authors' contributions: M. R. Downes designed the study. A. Hodgson, B. Xu and M. R. Downes gathered the immunohistochemistry data. J. Bayani and JMS. Bartlett performed the gene expression profiling. E. Olkhov-Mitsel, A. Hodgson, S. K. Liu, D. Vesprini, B. Xu and M. R. Downes reviewed and interpreted the data. E. Olkhov-Mitsel performed the RNA extraction and statistical analysis. E. Olkhov-Mitsel, A. Hodgson, S. K. Liu, D. Vesprini, B. Xu, J. Bayani, JMS. Bartlett and M. R. Downes contributed to drafting and review of the manuscript, and to approval of the final version.

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Declaration of Competing Interest: Dr. Downes has been an advisory board member for Astra Zeneca and Hoffman La Roche and has received speaker's honoraria from Astra Zeneca. All other authors have no disclosures.

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Tables

Table 1. Clinicopathological characteristics of the study cohort

	Total (N=40)	Fast relapsers, <2 years (N=25)	Slow relapsers, >2 years (N=15)	P-Value
Age (years)				
Mean	70	68	73	0.164
Range	33-88	33-82	60-88	
Sex				
Male	28 70.0%	20 80.0%	8 53.3%	0.081
Female	12 30.0%	5 20.0%	7 46.7%	
pT category				
pT2	1 2.5%	1 4.0%	0 0%	0.277
pT3	29 72.5%	16 64.0%	13 86.7%	
pT4	10 25.0%	8 32.0%	2 13.3%	
Margins				
Negative	27 67.5%	14 56.0%	13 86.7%	0.080
Positive	13 32.5%	11 44.0%	2 13.3%	
Lymphovascular invasion				
Absent	11 27.5%	3 12.0%	8 53.3%	0.009
Present	29 72.5%	22 88.0%	7 46.7%	
Lymph nodes				
Negative	25 62.5%	12 48.0%	13 86.7%	0.013
Positive	12 30.0%	11 44.0%	1 6.7%	
NA	3 7.5%	2 8.0%	1 6.7%	
Total lymph nodes retrieved				
Mean	13	11	16	0.094
Range	1-37	1-37	2-33	
CIS				
Absent	22 55.0%	12 48.0%	10 66.7%	0.332
Present	18 45.0%	13 52.0%	5 33.3%	
Invasive front inflammation				
Low	20 50.0%	17 68.0%	3 20%	0.008
High	20 50.0%	8 32.0%	12 80%	
Follow-up time (months)				
Mean	25	6	58	<0.001
Range	0-206	0-14	3-206	

Figure Captions

Figure 1. Kaplan-Meier estimates of fast relapse-free survival among patients with HGUC according to (A) CD3, (B) CD4, (C) CD8, (D) CD20, (E) CD68, (F) CD163, (G) FOXP3 and (H) PD-1 expression. The patients were dichotomized based on median IHC staining scores for each marker. The comparison of the KM curves between the high and low protein expression groups was based on the log-rank test (*P*-values shown).

Figure 2. (A)Volcano plot of differentially expressed genes between fast relapse versus non-fast relapse high grade urothelial bladder carcinoma (HGUC) cases. Three genes were significantly (log2 ratio >1 and $P < 0.05$) more abundant in non-fast relapse HGUC (red data points), and two genes were significantly more abundant in fast relapse HGUC (green data points)**.**
(B-F)Kaplan-Meier estimates of fast relapse-free survival among patients with HGUC according to (B) *CCL21*, (C) *CR2*, (D) *CD36*, (E) *HLA-DQA1* and (F) *KLRB1* expression. The patients were divided into high versus low based on median gene expression for each marker. The comparison of the KM curves between the high (above-median) and low (below-median) gene expression groups was based on the log-rank test (*P*-values shown).

Figure 1

Click here to access/download;Figure;FIGURE 1.jpg

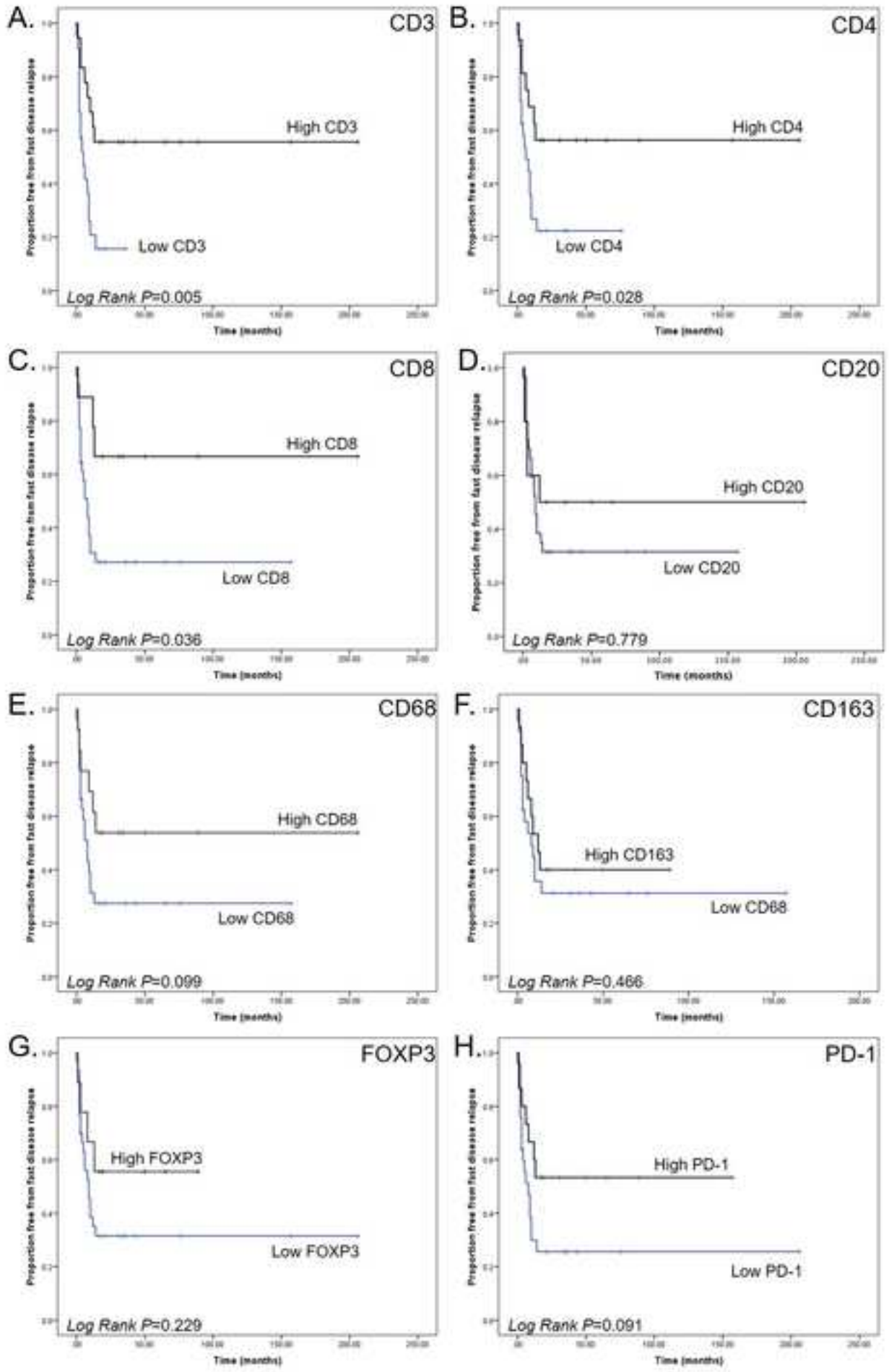

Figure 2