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BRIEF REPORT

Genomic and immunologic correlates of LAG-3 expression in cancer

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ABSTRACT

Immune checkpoint blockade leads to unprecedented responses in many cancers. Although currently available agents mostly target the PD-1 and CTLA-4 pathways, agents targeting the immune checkpoint protein LAG-3 are under active clinical development, and early clinical data show that *LAG-3* expression is a biomarker of response to LAG-3 blockade. To determine which cancers may benefit most from LAG-3 blockade, we performed a pan-cancer analysis of The Cancer Genome Atlas dataset to identify genomic and immunologic correlates of *LAG-3* expression. High mutation burden, and expression of exogenous virus (EBV, HPV) or endogenous retrovirus (*ERV3-2*), were associated with overexpression of *LAG-3* in multiple cancers. Although CD8+ T-cell marker (*CD8A*) and *LAG-3* were strongly co-expressed with each other and with *PD-L1* in most cancers, there were three notable exceptions: HPV+ head-neck squamous cell cancer, renal cell cancer, and glioblastoma. These results may have important implications for guiding development clinical trials of LAG-3 blockade.

Introduction

Immune checkpoint blockade leads to impressive and durable responses in multiple cancer types, and at present, both PD-1 and CTLA-4 blockade have been FDA approved for the treatment of several cancer types.[1–3] Apart from PD-1 and CTLA-4 pathways, there are several additional checkpoint genes, including *LAG3* (*LAG-3*), the BTLA-HVEM pathway, the TIM3-GAL9 pathway, *KIR3DL1* (*KIR), ADORA2A (A2aR), CD276* (*B7-H3*) and *VTCN1* (*B7-H4*), that can inhibit T-cell responses[4] and are being investigated as therapeutic targets for immune checkpoint blockade. Clinical trials utilizing anti-LAG-3 antibodies are underway to evaluate the safety and efficacy of LAG-3 blockade alone or in combination with PD-1 blockade with or without CTLA-4 blockade.

In a small cohort (N = 61) of melanoma patients, all of whom had progressed on prior treatment with PD-L1 and/or CTLA-4 targeting antibodies, a combination of LAG-3 blockade and PD-1 blockade was recently reported to have clinically significant anti-tumor activity, with 11.5% objective response rate and 49% disease control rate.[5] This finding suggests that *LAG-3* is an important immune checkpoint target in melanoma, and there is a need to identify which patients may benefit most from LAG-3 inhibition, either alone or in combination with PD-1 blockade.

In this initial melanoma study, the objective response rate to combination LAG-3 blockade and PD-1 blockade was 3.5-fold higher (18% vs 5%) in patients with immunohistochemistry-based LAG-3 expression ≥1% vs <1%, although both groups had previously progressed on PD-1 blockade.[5]

These observations suggest that *LAG-3* expression is a biomarker of response to LAG-3 blockade, like *CD274* (*PD-L1*) expression is in case of PD-1 blockade.[6] Therefore, we performed a pan-cancer analysis of The Cancer Genome Atlas (TCGA) to identify genomic and immunologic correlates of *LAG-3* expression, which may potentially help predict response to LAG-3 blockade.

Materials and methods

RNA-seq data of tumors for all cancer types in TCGA were obtained from the Broad Genome Data Analysis Center (http://gdac.broadinstitute.org) and the TCGA Data Portal (https://tcga-data.nci.nih.gov/docs/publications/tcga/), and were median adjusted and log_2 transformed in the same way as previously described.[7] *ERBB2* focal copy number data and *ESR1* mRNA expression data from the Broad Genome Data Analysis Center were used to classify breast cancer samples into clinical subtypes (ER+/HER2−, ER−/HER2−, HER2+), and these subtypes were analyzed separately. Hyper-mutation status,[8] Epstein-Barr virus (EBV) status,[9] Hepatitis B and C (HBV and HCV) status,[10] and *ERV3-2* expression data[11] were obtained from recently published studies. Human papillomavirus (HPV) status was obtained from auxiliary clinical files from the TCGA Data Portal. Wilcoxon rank-sum test was used for all pairwise comparisons. Spearman Rho was used for all correlations, and *P*-values were calculated using either the exact permutation distributions (for small sample sizes) or large-sample approximations. All *P*-values are from two-sided

tests, and $P < .05$ was used as the threshold for statistical significance. Multiple hypothesis testing correction was performed using Benjamini-Hochberg procedure. A set of 29 clear cell renal cell carcinoma (RCC) samples from Rutgers Cancer Institute of New Jersey (CINJ), obtained with consent under an IRB approved Total Cancer Care® Protocol through the Oncology Research Information Exchange Network (ORIEN) (Pro20150001762), that underwent whole exome sequencing and RNA sequencing, was used for validation. De-identified RNA sequencing data from this group were analyzed in this study.

Results

LAG-3 is co-expressed with CD8A and PD-L1 in most cancer types

Analysis of *LAG-3* expression in TCGA dataset showed a wide range of expression among different cancer types (Figure 1). The highest median expression was observed in diffuse large B-cell lymphoma. Multiple solid tumors, including melanoma, cervical cancer, and head and neck squamous cell cancer had considerably
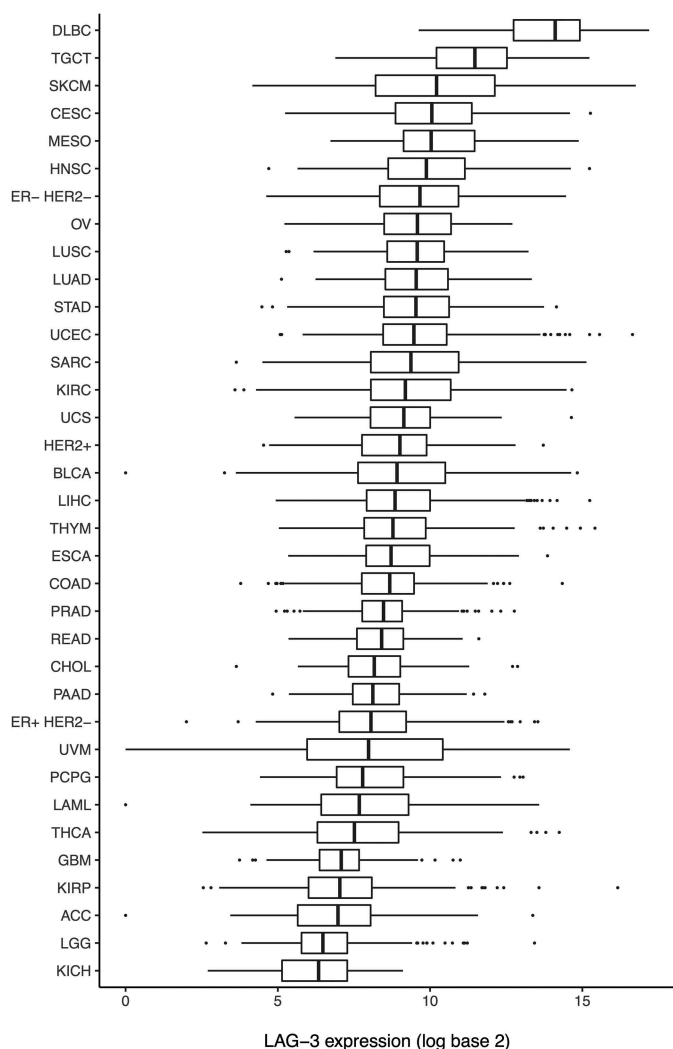
high expression of *LAG-3*. Interestingly, outlier high expression of *LAG-3* was observed in a subset of cases in several cancer types, including endometrial cancer, liver cancer, and RCC.

A pan-cancer analysis to identify genes whose expressions correlated (Spearman Rho > 0, P < .05) or anti-correlated (Spearman Rho < 0, P < .05) with *LAG-3* expression showed that the number of cancer types in which a gene's expression correlated with *LAG-3* expression (minus the number of cancer types in which the gene's expression anti-correlated with *LAG-3* expression) had a bimodal distribution (Supplementary Figure 1). Pathway enrichment analysis of the genes in the second mode (i.e. genes whose expressions were correlated with *LAG-3* expression in unusually high numbers of cancer types) using the ToppGene suite[12] showed that these genes are involved in adaptive immune system, innate immune system, cytokine signaling, and interferon-gamma signaling (Supplementary Figure 1).

CD8+T-cells infiltrates in cancer are correlated with local immune activation and response to immune checkpoint therapy.[13] To evaluate whether *LAG-3* expression was associated with CD8$^+$ T-cell infiltration, we tested for correlation between *LAG-3* expression and expression of the cytotoxic T-cell marker *CD8A*. We found that *CD8A* expression was strongly correlated (Spearman Rho > 0.5) with *LAG-3* expression in almost every cancer type (Figure 2(a)), suggesting that tumors with strong CD8$^+$ T-cell infiltration tend to have high *LAG3*-expression. This strong correlation may be due to the expression of *LAG-3* on a subset of CD8$^+$ T cells.[14-17] *CD8A* expression was also correlated (although less strongly, Spearman Rho < 0.5) with *PD-L1* expression in most cancer types (Figure 2(b)), suggesting that the presence of cytotoxic T-cells correlates with both *LAG-3* and *PD-L1* expression. In fact, *CD8A, PD-L1,* and *LAG-3* were co-expressed in most cancer types (Figure 2(c)), suggesting that the combination of LAG-3 blockade and PD-1 or PD-L1 blockade is a reasonable strategy. Similar co-expression was also observed for *CD8A, CTLA-4,* and *LAG-3* (Supplementary Figure 2).

CD8A has a stronger correlation with LAG-3 than PD-L1 in renal cancers and glioblastoma

RCC (KIRC, KIRP, KICH) and glioma (GBM, LGG) were notable exceptions to the above trend (Figure 2(c)). In RCC (Figure 2(c,d)), the correlation between *CD8A* and *LAG-3* was much stronger than the relatively weak correlation between *CD8A* and *PD-L1*. To validate these findings, we analyzed an independent set of 29 clear cell RCC samples from CINJ that had undergone RNA sequencing. Here again there was a strong correlation between *LAG-3* and *CD8A* expression (Figure 2(e)), while there was no correlation between *PD-L1* and *CD8A* expression (Figure 2(e)), confirming the findings in TCGA.

In TCGA glioma data (Figure 2(c,f)), *CD8A* expression was correlated with *LAG-3* expression (but not *PD-L1* expression) in glioblastoma multiforme and *PD-L1* expression (but not *LAG-3* expression) in low-grade glioma, suggesting that LAG-3 blockade and PD-1 blockade may, respectively, be appropriate for tumors with CD8$^+$ T-cell infiltration in glioblastoma multiforme and low-grade glioma. A Phase I clinical trial of LAG-3 blockade with or without PD-1 blockade in glioblastoma multiforme is currently in progress (NCT02658981), and it will be
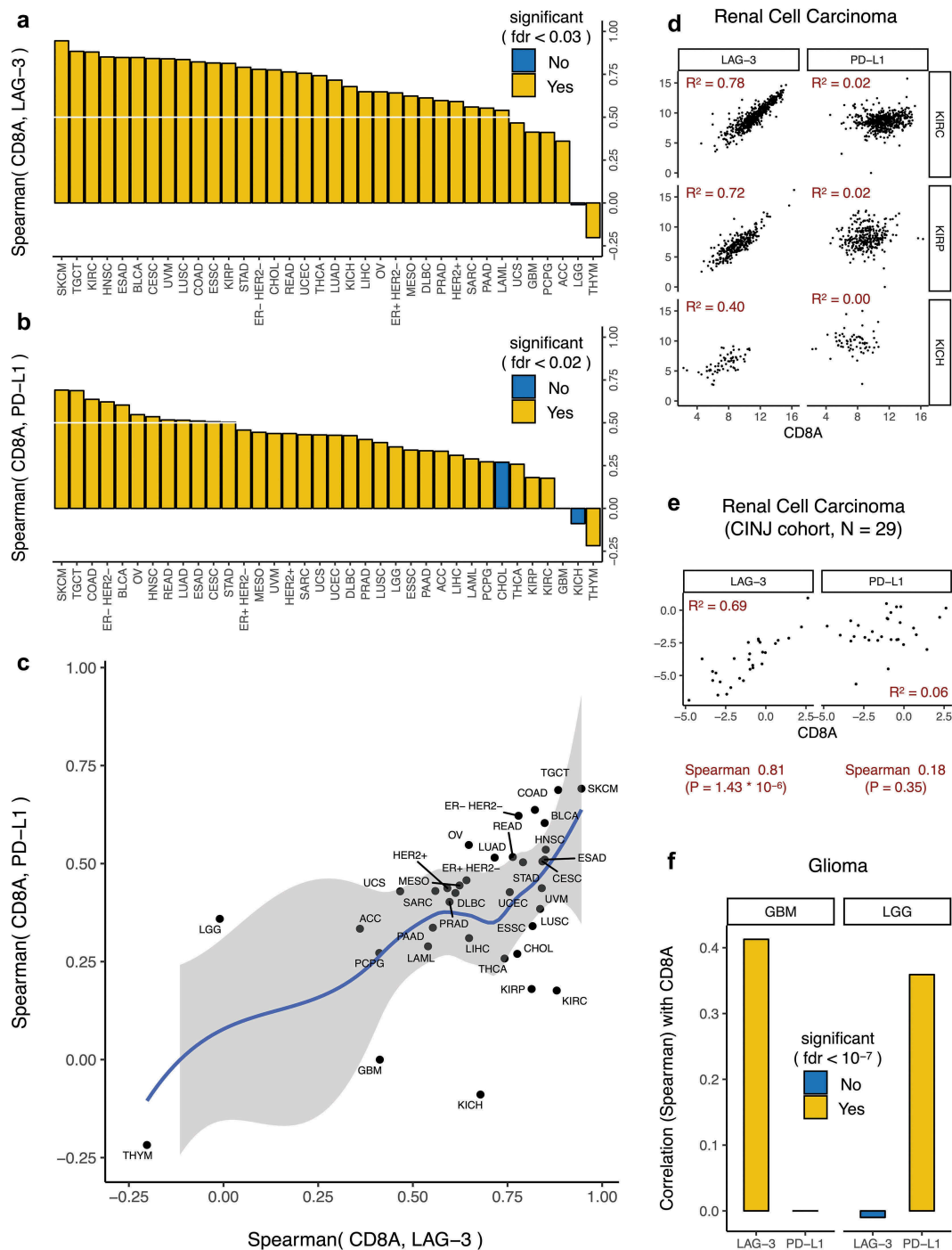

Figure 1. Expression of *LAG-3* in various cancer types.
Cancer type acronyms are standard TCGA abbreviations (https://gdc.cancer.gov/resources-tcga-users/tcga-code-tables/tcga-study-abbreviations)

Figure 2. Immunologic correlates of *LAG-3* expression in all cancer types.

[a] Correlation between expressions of *CD8A* and *LAG-3*. [b] Correlation between expressions of *CD8A* and *PD-L1* (the white line marks Spearman Rho = 0.5). [c] Comparison of the above two correlations, with LOESS curve in blue and 95% confidence interval in gray. [d] Expressions of *LAG-3* and *PD-L1* as a function of *CD8A* expression in renal cell carcinoma (R^2 for linear fit specified). [e] Independent confirmation of the renal cell carcinoma results using data from the CINJ cohort (a subset of the larger ORIEN dataset). The axes are RSEM normalized mRNA expression. [f] Correlation between expressions of *CD8A, LAG-3*, and *PD-L1* in glioma. Cancer type acronyms are standard TCGA abbreviations (https://gdc.cancer.gov/resources-tcga-users/tcga-code-tables/tcga-study-abbreviations). fdr = false discovery rate.

interesting to see whether LAG-3 blockade shows promising clinical activity in tumors with CD8+ T-cell infiltration.

Association between *LAG-3* expression and tumor mutation burden

We recently showed[8] that in eight solid cancer types, there exists a non-synonymous mutation burden threshold (iCAM), such that tumors above this threshold (iCAM+) show RNA-seq based evidence of immune activation and PD-1 or CTLA-4 pathway upregulation in TCGA dataset. Tumors classified as iCAM+ were more sensitive to PD-1 blockade and CTLA-4 blockade in publicly available datasets of melanoma, lung, and colorectal cancers compared with tumors below this threshold (iCAM−).[8] To determine whether *LAG-3* expression is also associated with mutation burden, we evaluated the relative

expression of *LAG-3* in high mutation burden (iCAM+) vs low mutation burden (iCAM−) tumors in these eight solid cancer types. In all eight cancer types, iCAM+ tumors showed significant overexpression of *LAG-3* compared with iCAM− tumors (Figure 3(a)), suggesting that high mutation burden, as identified using the iCAM threshold, may also be a biomarker of response to LAG-3 blockade.

Similar comparison for other less-studied checkpoints (Figure 3(b)) showed that unlike the PD-1 pathway, CTLA-4 pathway, and *LAG-3*, other checkpoints were overexpressed in iCAM+ tumors in a much more cancer type-specific manner. For example, the BTLA-HVEM pathway was upregulated in iCAM+ tumors only in endometrial cancer (UCEC), and the TIM3-GAL9 pathway was upregulated in iCAM+ tumors only
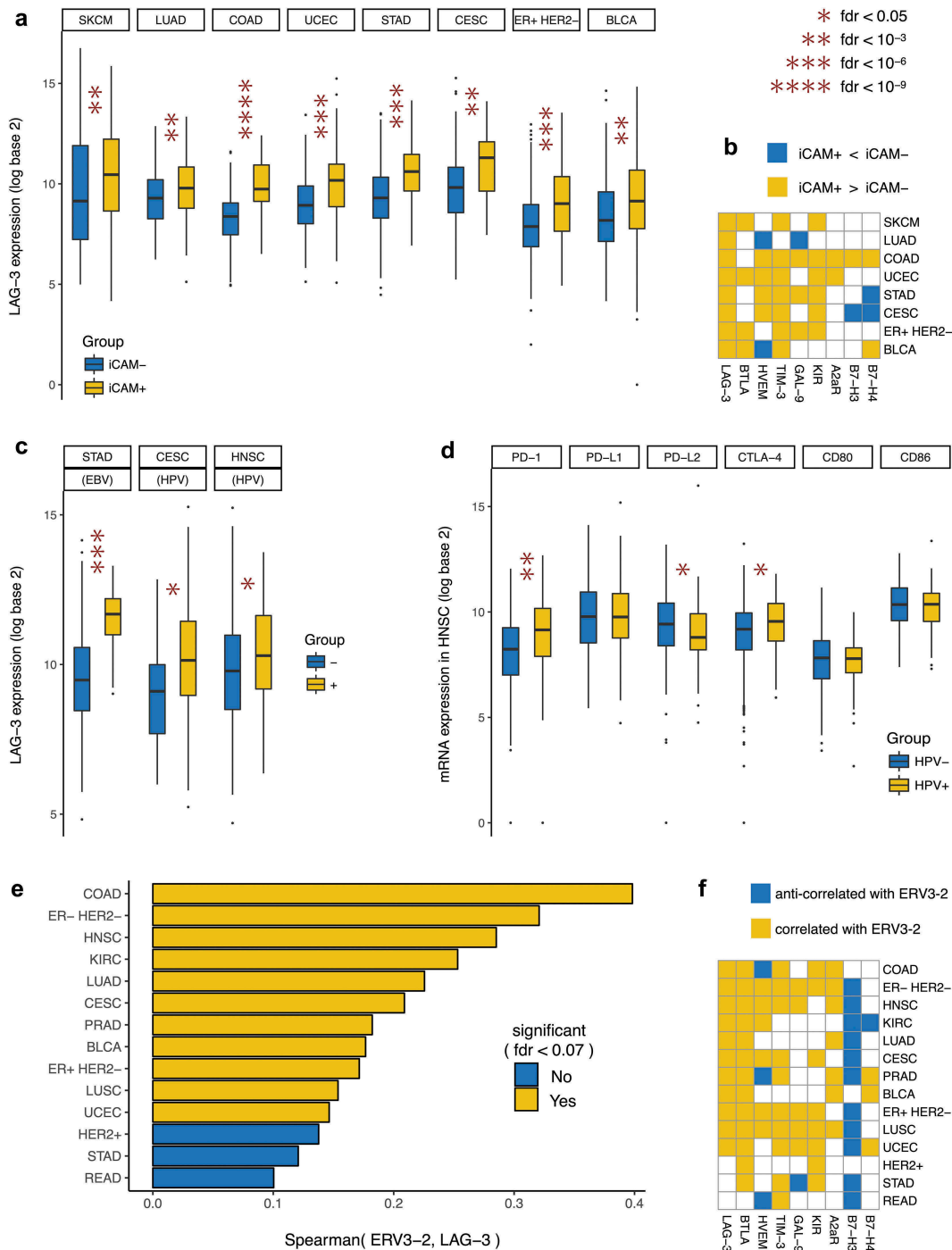

Figure 3. Genomic correlates of *LAG-3* expression in selected cancer types.

[a] *LAG-3* expression in high mutation burden (iCAM+) and low mutation burden (iCAM−) tumors. [b] Expression of some other immune checkpoint genes in iCAM+ and iCAM− tumors. [c] *LAG-3* expression in tumors positive and negative for EBV or HPV. [d] Expression of immune checkpoint genes in PD-1 pathway and CTLA-4 pathway in head and neck squamous cell cancer by HPV status. [e] Correlation between expressions of *ERV3-2* and *LAG-3*. [f] Correlation between expressions of *ERV3-2* and some other immune checkpoint genes. Cancer type acronyms are standard TCGA abbreviations (https://gdc.cancer.gov/resources-tcga-users/tcga-code-tables/tcga-study-abbreviations). fdr = false discovery rate.

in colon (COAD), gastric (STAD), and ER+ HER2– breast cancer.

LAG-3 is overexpressed in virally mediated tumors

Expression of exogenous viruses, including the presence of EBV in gastric cancer,[18,19] and HPV in cervical cancer[20] and head and neck squamous cell cancer[20] has also been associated with evidence of immune activation. EBV expression in gastric cancer,[7] NK/T-cell lymphoma,[21] and Hodgkin's disease[22] has been associated with response to PD-1 blockade. To evaluate the relationship between LAG-3 expression and viral infection in tumor, we tested for association between LAG-3 expression and EBV or HPV status in these cancer types. EBV+ tumors in gastric cancer (STAD), and HPV+ tumors in cervical (CESC) and head and neck squamous cell (HNSC) cancer showed significant overexpression of LAG-3 compared with EBV– and HPV– tumors of these cancer types (Figure 3(c)). While the PD-1 and CTLA-4 pathways were also significantly upregulated in EBV+ gastric cancer[7] and HPV+ cervical cancer (Supplementary Figure 3), this was not the case in HPV+ head and neck squamous cell cancer. In head and neck squamous cell cancer (Figure 3(d)), neither the ligands of PDCD1 (PD-1) nor the ligands of CTLA-4 were overexpressed in HPV+ tumors compared with HPV– tumors, which makes the overexpression of LAG-3 especially interesting. Unlike EBV+ and HPV+ tumors (Supplementary Figure 3), HBV+ and HCV+ tumors in liver cancer showed no evidence of CD8$^+$ T-cell activation and overexpression of LAG-3 or other immune checkpoint genes (Supplementary Figure 4).

Association between LAG-3 expression and expression of potentially immunogenic ERV

The expression of normally silenced endogenous retroviral RNAs has been shown to be a potential mechanism of activation of innate immune signaling.[23] We recently showed that expression of the potentially immunogenic endogenous retrovirus ERV3-2 is correlated with overall immune infiltration in 14 solid cancer types.[24] To determine whether ERV expression is associated with LAG-3 expression, we tested for correlation between ERV3-2 expression and LAG-3 expression in these 14 cancer types. In 11 of the 14 cancer types, ERV3-2 expression was significantly (P < .05) correlated with LAG-3 expression (Figure 3(e)), suggesting that ERV3-2 expression may be a predictor of response to LAG-3 blockade in these cancer types. In addition to LAG-3, ERV3-2 expression was also associated with the upregulation of PD-1 and CTLA-4 pathways in most of these cancer types,[24] suggesting the coordinated upregulation of multiple immune checkpoint genes in ERV-expressing tumors. In contrast, ERV3-2 expression was associated with overexpression of other checkpoint genes in a more cancer type-specific manner, such as the BTLA-HVEM pathway in only six cancer types and the TIM3-GAL9 pathway in only five cancer types (Figure 3(f)). A pancancer analysis to identify ERVs whose expressions correlated (Spearman Rho > 0, P < .05) or anti-correlated (Spearman Rho < 0, P < .05) with LAG-3 expression showed that ERV3-2 expression is correlated with LAG-3 expression in more cancer types than any other ERV (Supplementary Figure 5). Association

between ERV3-2 expression and response to LAG-3 blockade with or without PD-1 blockade in these cancer types should be investigated in clinical trials.

Discussion

This pan-cancer analysis of LAG-3 expression shows that for most, but not all, cancers, LAG-3 expression follows the same pattern as PD-L1 expression and is similarly associated with expression of marker of cytotoxic T-cell activity.[15,25] Thus, for the most part, our data suggest that LAG-3 blockade will likely benefit the same subset of cancers already shown to benefit from PD-1 pathway blockade, and suggests that clinical trials combining PD-1 and LAG-3 antibodies are rational approaches in most cancer types. The highest expression of LAG-3 is seen in B-cell lymphoma (Figure 1), and trials are currently underway for LAG-3 blockade alone and in combination with PD-1 pathway inhibitors in B-cell lymphoma (NCT02061761).

However, our analysis also showed that there are some cancers, such as RCC, where the correlation between LAG-3 and CD8A expression is much stronger than that seen for PD-L1 and CD8A. This correlation may be due to the expression of LAG-3 in a subset of CD8$^+$ T cells.[14-17] PD-L1 can be expressed both by tumor cells and immune cells, such as macrophages, in the tumor microenvironment. A lack of correlation between PD-L1 and CD8A in the setting of a high correlation between LAG-3 and CD8A in some cancers suggests that PD-L1 may not be associated with T-cell infiltrates marked by LAG-3 in these cancers. This novel observation suggests that LAG-3 blockade may have substantial clinical activity in RCC with strong CD8$^+$ T-cell infiltration, possibly more than from PD-1 pathway blockade. This finding also suggests that LAG-3 inhibition may be effective in a subset of patients that are not responsive to PD-1/PD-L1 blockade in ccRCC. While a Phase II clinical trial of a combination of LAG-3 blockade and PD-1 blockade is already in progress in RCC (NCT02996110), our data suggest clinical trials exploring the efficacy of LAG-3 blockade monotherapy may also be warranted in RCC. Similarly, CD8A expression in glioblastoma was more strongly associated with LAG-3 expression than PD-L1 expression, suggesting that clinical trials of LAG-3 blockade should also be investigated in these aggressive cancers. Phase I clinical trial of LAG-3 blockade in glioblastoma is currently in progress (NCT02658981). As new LAG-3 inhibitors enter clinical trials, our data suggest that RCC and glioblastoma may be populations of special interest for these agents.

Since protein expression data of LAG-3 are unavailable in the TCGA dataset, the analysis presented in this paper is limited to mRNA expression data of LAG-3. However, the human protein atlas database (https://www.proteinatlas.org/ENSG00000089692-LAG3/tissue) shows that protein expression of LAG-3 is observed in appendix, lymph node, and tonsil – tissues that also have relatively high mRNA expression of LAG-3 – suggesting a concordance between mRNA expression and protein expression of LAG-3.

Anomalous results were observed in case of thymoma (THYM, Figure 2(a–c)), where CD8A expression was anticorrelated with both LAG-3 expression and PD-L1 expression. However, it should be noted that while CD8A expression is

a surrogate for CD8$^+$ T cell infiltration in most tissues, that may or may not be the case in thymoma. Unlike other tissues, thymus normally makes and stores CD8$^+$ T cells, so the *CD8A* expression may represent the normally present CD8$^+$ T cells in thymus, or real CD8$^+$ T cell infiltration in response to cancer cells in thymoma, or some combination of both. Thus, interpretation of *CD8A* expression in thymic cancers is difficult, and therefore we cannot draw any conclusion in case of thymoma.

Our findings also suggest that increased mutation burden, as identified by the iCAM threshold,[8] will likely be associated with response to LAG-3 blockade, similar to its known association with response to PD-1 pathway blockade. Phase II clinical trials of a combination of LAG-3 blockade and PD-1 blockade are currently in progress in non-small cell lung (NCT02750514), colon (NCT02060188), and gastric cancers (NCT02935634). It will be of great interest to see whether mutation burden is correlated with response, and whether iCAM+ tumors have high response rates in these trials. Similar trials are also warranted in skin melanoma, endometrial, cervical, bladder, and ER+ HER2− breast cancer, where we predict that high mutation burden (iCAM+) tumors may also be responsive to LAG-3 blockade.

Our results suggest that certain virally infected cancers, including EBV+ gastric cancers, and HPV+ cervical and head-and-neck cancers, also have high expression of *LAG-3*. A Phase I/II clinical trial of a combination of LAG-3 blockade and PD-1 blockade is currently in progress in virally mediated solid cancers (NCT02488759). Based on *LAG-3* expression, our data would suggest that EBV+ tumors, in particular, may have great benefit from LAG-3 blockade, either alone or in combination with PD-1 pathway blockade. HPV+ head and neck squamous cell cancers is another group where LAG-3 blockade may be of particular benefit.

Similarly, *LAG-3* expression is also correlated with abnormal expression of the potentially immunogenic endogenous retrovirus *ERV3-2* in several cancer types. We previously showed that *ERV3-2* expression is associated with response to PD-1 blockade in clear cell RCC,[24] and these data suggest that LAG-3 blockade may also be effective in *ERV3-2*–expressing clear cell RCC.

Overall, analysis of TCGA data shows that *LAG-3* expression is for the most part associated with known biomarkers of response to PD-1 pathway blockade, including tumor mutation burden, viral infection, and expression of immunogenic endogenous retrovirus. However, this analysis also suggests that targeting LAG-3 may be more effective than PD-1 pathway blockade in subsets of RCC, glioblastoma, and HPV+ head and neck cancers. Data from proposed and ongoing clinical trials are needed to validate these hypotheses.

Disclosure of potential conflicts of interest

AP: No conflicts; JAR: No conflicts; EAS: Clinical trial research support from Astellas/Medivation; GB: No conflicts; SG: Consulted for Novartis, Roche, Foundation Medicine, Foghorn Therapeutics, and Inspirata; Owns equity in and has licensed patents to Inspirata; Spouse is an employee of Merck and has equity in Merck.

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