Development of a LAG-3 immunohistochemistry assay for melanoma

Lori Johnson,[1] Bryan McCune,[2] Darren Locke,[3] Cyrus Hedvat,[3] John B Wojcik,[3] Caitlin Schroyer,[1] Jim Yan,[1] Krystal Johnson,[2] Angela Sanders-Cliette,[2] Sujana Samala,[2] Lloye M Dillon,[3] Steven Anderson,[2] Jeffrey Shuster [ID] [1]

ABSTRACT

Aims A robust immunohistochemistry (IHC) assay was developed to detect lymphocyte-activation gene 3 (LAG-3) expression by immune cells (ICs) in tumour tissues. LAG-3 is an immuno-oncology target with demonstrable clinical benefit, and there is a need for a standardised, well-characterised assay to measure its expression. This study aims to describe LAG-3 scoring criteria and present the specificity, sensitivity, analytical precision and reproducibility of this assay.

Methods The specificity of the assay was investigated by antigen competition and with *LAG3* knockout cell lines. A melanin pigment removal procedure was implemented to prevent melanin interference in IHC interpretation. Formalin-fixed paraffin-embedded (FFPE) human melanoma samples with a range of LAG-3 expression levels were used to assess the sensitivity and analytical precision of the assay with a ≥1% cut-off to determine LAG-3 positivity. Interobserver and intraobserver reproducibility were evaluated with 60 samples in intralaboratory studies and 70 samples in interlaboratory studies.

Results The LAG-3 IHC method demonstrated performance suitable for analysis of LAG-3 IC expression in clinical melanoma samples. The pretreatment step effectively removed melanin pigment that could interfere with interpretation. LAG-3 antigen competition and analysis of *LAG3* knockout cell lines indicated that the 17B4 antibody clone binds specifically to LAG-3. The intrarun repeatability, interday, interinstrument, interoperator and inter-reagent lot reproducibility demonstrated a high scoring concordance (>95%). The interobserver and intraobserver reproducibility and overall interlaboratory and intralaboratory reproducibility also showed high scoring concordance (>90%).

Conclusions We have demonstrated that the assay reliably assesses LAG-3 expression in FFPE human melanoma samples by IHC.

INTRODUCTION

Immune checkpoint inhibitor–based therapies have greatly improved clinical outcomes across multiple disease settings,[1 2] including advanced melanoma,[3–5] non-small cell lung cancer,[6 7] squamous cell carcinoma of the head and neck[8 9] and urothelial carcinoma,[10 11] among others. However, given the multiple mechanisms of immune evasion used by cancer cells, inhibition of a single immune checkpoint, such as programmed death-1 (PD-1), may not be sufficient to overcome immune

suppression.[12 13] Novel immuno-oncology (I-O) combinations, including dual checkpoint inhibition, may be necessary to enhance efficacy and to improve the durability of patient responses.

Lymphocyte-activation gene 3 (LAG-3, CD223) is a cell-surface molecule expressed on activated CD4+ and CD8+ T cells, as well as other immune cells (ICs) including regulatory T cells, natural killer cells, B cells, macrophages and dendritic cells, and is under investigation as an I-O therapy target.[13–17] The interaction of LAG-3 with its ligands, the major histocompatibility complex II and fibrinogen-like protein 1, recently discovered as a LAG-3 ligand, initiate an inhibitory signal.[13 18 19] This signal can impair T-cell function, activation and proliferation, decrease production of and response to proinflammatory cytokines and decrease the development of memory T cells.

Preclinical data indicate that simultaneous activation of the LAG-3 and PD-1 pathways in tumor-infiltrating lymphocytes results in greater T-cell exhaustion than either pathway alone, and dual inhibition of these pathways may improve T-cell function and increase immune response.[20] Furthermore, combined therapy with anti–LAG-3 and anti–PD-1 agents in fibrosarcoma and colorectal adenocarcinoma mouse models resulted in synergistic antitumor activity.[16] The clinical efficacy of combining relatlimab, an anti–LAG-3 antibody, with nivolumab, an anti–PD-1 agent, was previously demonstrated in patients with previously untreated metastatic or unresectable melanoma by the phase II/III RELATIVITY-047 clinical trial (NCT03470922).[21] RELATIVITY-047 demonstrated superior progression-free survival (PFS) for relatlimab combined with nivolumab versus nivolumab monotherapy, regardless of LAG-3 expression.[21] In March 2022, the US Food and Drug Administration (FDA) approved nivolumab and relatlimab-rmbw (Opdualag; relatlimab-rmbw is the name used when referring to the approval by FDA: as per the agency naming guidance, the naming convention for biological products licensed under the Public Health Service Act is a proper name consisting of a core name and an FDA-designated suffix[22]) for adult and paediatric patients ≥12 years of age with unresectable or metastatic melanoma.[23]

A robust immunohistochemistry (IHC) assay was developed to detect LAG-3 expression by ICs. The assay was used to stratify patients enrolled in RELATIVITY-047, based on the percentage of LAG-3–positive ICs with a morphological resemblance to

lymphocytes relative to all nucleated cells within the tumour region (tumour cells (TCs), intratumoral stroma and peritumoral stroma (the band of stromal elements directly contiguous with the outer tumor margin)) in samples containing ≥100 viable TCs. This assay is also being used in several ongoing clinical trials evaluating relatlimab. This study presents the specificity, sensitivity, analytical precision and reproducibility of this assay as an aid to determine LAG-3 expression in melanoma patients using a ≥1% IC expression threshold.

MATERIALS AND METHODS
Principles of the LAG-3 IHC assay
The LAG-3 IHC assay was developed using a mouse monoclonal antibody clone 17B4 that was made to a synthetic peptide corresponding to the 30-amino acid extra-loop of the first immunoglobulin domain of LAG-3, GPPAAAPGHPLAPGPHPAAP SSWGPRPRRY.[24] The assay was performed on formalin-fixed paraffin-embedded (FFPE) tissue sections mounted on glass slides and included pretreatment to remove endogenous melanin that could interfere with interpretation of LAG-3 staining. Following pretreatment, slides were stained and processed using the 17B4 primary antibody on a Leica BOND-III autostainer (Leica Biosystems, Buffalo Grove, Illinois, USA).

Materials
Tissue specimens
FFPE melanoma specimens and control tonsil tissues were obtained from commercial vendors (Boca Biolistics, Pompano Beach, FL, USA; BioIVT, Westbury, New York, USA; and Avaden Biosciences, Seattle, Washington, USA). Sections were cut from each tissue block at 4 µm thickness, placed on positively charged slides and dried for 1 hour at 60°C±2°C. Excepting sample stability studies, all cut sections were tested within 2 months of sectioning.

Antibodies
All experiments were performed with monoclonal LAG-3 antibody 17B4 preparations manufactured from hybridoma cultures for Labcorp, except for analysis of clustered regularly interspaced short palindromic repeats (CRISPR)-engineered LAG-3 knockout cell lines, for which a commercially available LAG-3 17B4 antibody was obtained from LSBio (Cat. no. LS-C18692) or as otherwise noted in the text.[24] For precision studies, three independent lots of antibody were produced from the 17B4 hybridoma. The working concentration of the LAG-3 17B4 antibody was 2.5 µg/mL. The negative control antibody, mouse monoclonal immunoglobulin G1 clone MOPC-21, was obtained from Leica Biosystems (Cat. no. PA0996). Further details on the staining and melanin removal procedures are described in the online supplemental material and online supplemental table 1.

Melanin scoring
To determine the efficacy of the melanin removal step of the protocol, the amount of melanin pigment in the tumour region was scored on a scale of 0 to 4+. Definitions for melanin pigment scoring expected on melanoma tissue-stained slides and indications for the evaluability of the melanin interpretation in LAG-3 IHC assay scoring are provided in online supplemental table 2.

LAG-3 scoring
An overview of the LAG-3 scoring method is provided in online supplemental figure 1. Evaluation criteria for staining intensity of LAG-3–positive ICs consisted of weak (1+), moderate (2+)

and strong (3+) LAG-3–positive staining (online supplemental table 3). In addition to cell-surface expression, LAG-3 protein is also retained in intracellular compartments.[25] Thus, LAG-3 IC positivity was quantified in cells that morphologically resembled lymphocytes with punctate (perinuclear and/or Golgi pattern), cytoplasmic and/or membranous LAG-3 staining of any intensity above background (online supplemental figure 2). LAG-3–positive IC content in the tumour region was visually estimated by microscopic examination by the study pathologists, following group alignment using a reference slide set. An H&E-stained slide for each melanoma sample tested was reviewed by a pathologist to identify the overall tumour region and confirm the presence of ≥100 TCs. Results were reported as the percentage of LAG-3–positive ICs relative to all nucleated cells (ICs (lymphocytes and macrophages), stromal cells and TCs) within the overall tumour region. The tumour region included TCs, intratumoral stroma and peritumoral stroma (the band of stromal elements directly contiguous with the outer tumour margin). Normal and/or adjacent uninvolved tissues were not included (online supplemental figure 3). The scoring scale was (in %) 0, 1, 2, 3, 4, 5, 10 and further increments of 10 up to 100. Samples with LAG-3-positive IC percentage scores of ≥1% were reported as LAG-3–positive.

The methods for the generation of CRISPR-engineered LAG-3 knockout cell lines, peptide inhibition assay, precision study measurements and reproducibility within the same laboratory and across laboratories, and stability experiments are provided in the online supplemental material.

RESULTS
Components of the LAG-3 IHC assay
Primary antibody concentration and incubation times for assay components were optimised for appropriate positive staining, staining intensity and overall staining quality of LAG-3 while minimising non-specific background staining. Antibody concentrations of 1.25 µg/mL, 2.5 µg/mL, 3.0 µg/mL and 3.5 µg/mL were evaluated, and 2.5 µg/mL was determined to be the optimal concentration.

Detection of LAG-3 in tissues using the 17B4 clone antibody
To investigate the ability of the LAG-3 IHC assay to detect LAG-3 IC expression in human FFPE tissue samples, the assay was used to stain LAG-3 in commercially procured human tonsil tissue. We hypothesised that if the LAG-3 IHC assay detected LAG-3 IC expression, then staining would be present in lymphocytes, but not in non-immune regions, such as the crypt epithelium. Staining of the tonsil tissues using the LAG-3 IHC assay revealed membranous/cytoplasmic staining of LAG-3 in lymphocytes in germinal centre and interfollicular regions, but no LAG-3 staining in the crypt epithelium (figure 1A). Additionally, no staining was observed in the slide stained with the mouse IgG isotype control.

The LAG-3 IHC assay was developed to include attenuation of melanin staining from FFPE sections prior to IHC and to minimise the impact of melanin pigment on interpretation of the assay. Examples of different levels of melanin pigmentation are shown in online supplemental figure 4. The efficacy of melanin removal from tissue samples using the melanin removal procedure is shown in figure 1B,C. All melanoma tissue samples selected for further investigation had acceptable negative control staining and melanin pigmentation ≤1+. LAG-3 staining was consistent in bleached and unbleached serial sections from the same tissue block (data not shown).
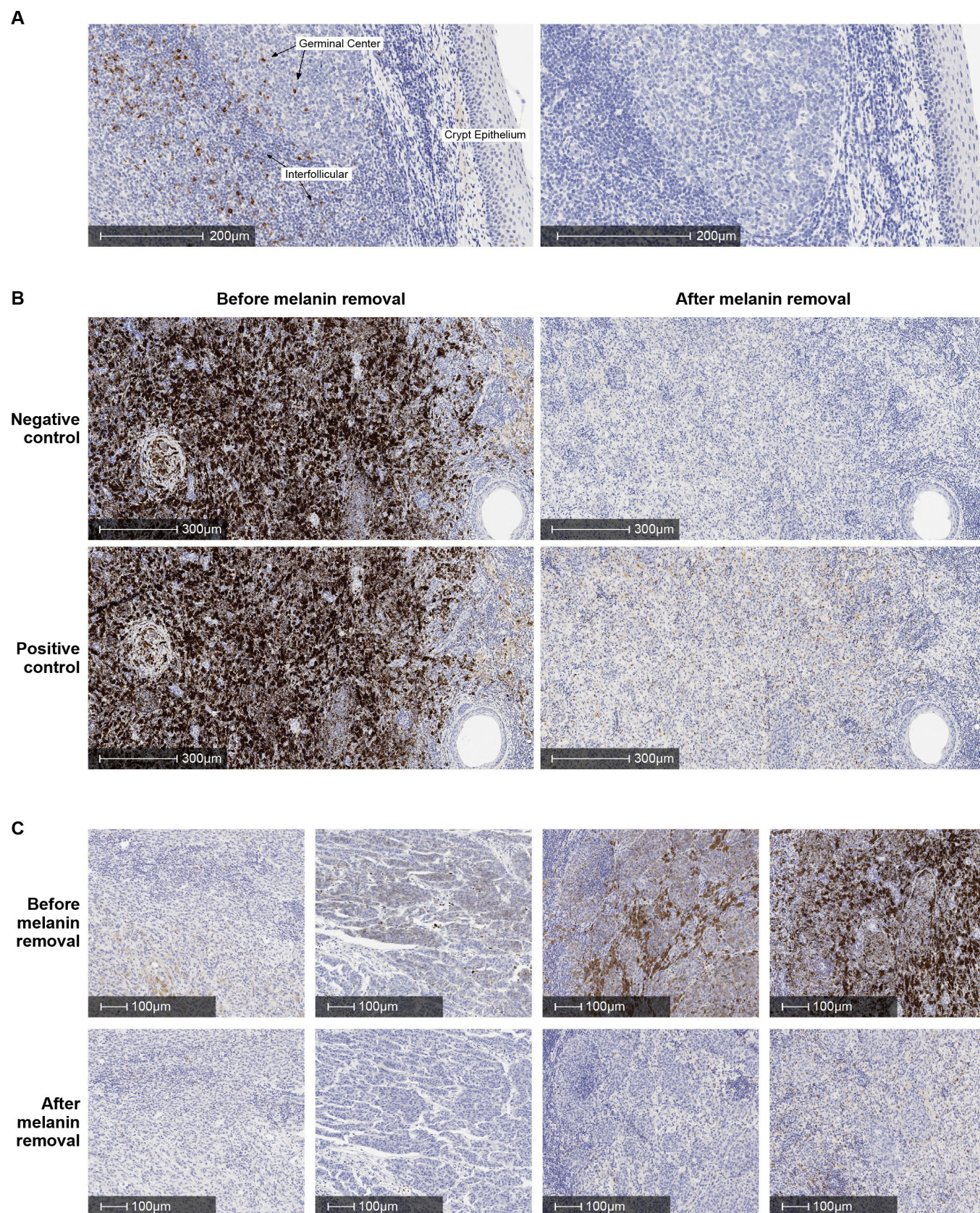
Figure 1   Identification of LAG-3 in human tissues using the LAG-3 IHC assay. (A) Detection of LAG-3 in human tonsil tissue. Left-hand image depicts LAG-3 staining pattern in tonsil tissue showing moderate-to-strong plasma membrane/cytoplasmic staining in lymphocytes in germinal centres and interfollicular region. The crypt epithelium is negative. No staining is seen with negative reagent control (right-hand image). (B) Staining of FFPE melanoma samples with negative reagent control (upper) or LAG-3 antibody (lower) before (left) and after (right) melanin removal procedure at ×10 magnification. (C) Examples of LAG-3 staining in FFPE melanoma samples before (upper) and after (lower) the melanin removal procedure at ×20 magnification. FFPE, formalin-fixed paraffin-embedded; IHC, immunohistochemistry; LAG-3, lymphocyte-activation gene 3.
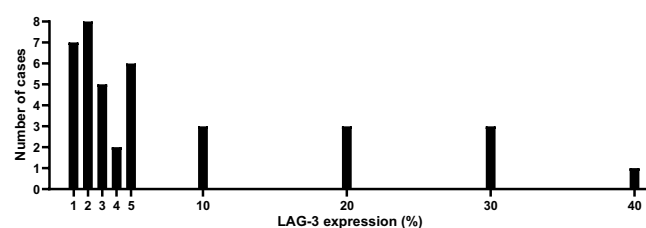
Figure 2 Detection of a range of LAG-3 expression levels using the LAG-3 IHC assay. Bar chart showing scoring distribution across LAG-3–positive samples (defined as those with LAG-3–positive IC content ≥1%) from a set of 100 commercially procured human FFPE melanoma specimens. Of the 100 samples, 38 were LAG-3–positive and 62 were LAG-3–negative. FFPE, formalin-fixed paraffin-embedded; IC, immune cell; IHC, immunohistochemistry; LAG-3, lymphocyte-activation gene 3.

Specificity and sensitivity of the LAG-3 IHC assay

To investigate the specificity of the LAG-3 IHC assay, the *LAG3* gene was disrupted by CRISPR-mediated mutagenesis in COV434 cell lines. In total, three pooled cell lines were derived, each with differing levels of *LAG3* knockout (out-of-frame indel frequency=71.02% in Cr1, 62.07% in Cr2 and 65.74% in Cr3) (online supplemental figure 5A). The LAG-3 expression of these cell lines was compared with parental COV434 cells to investigate the specificity of the LAG-3 IHC assay. LAG-3 staining in parental COV434 cells was markedly higher than each of the three *LAG3* knockout cell lines, which each had staining consistent with anticipated levels of residual LAG-3 expression based on the frequency of alterations determined by next-generation sequencing (online supplemental figure 5B). These data suggest that the LAG-3 IHC assay is specific for the detection of LAG-3 protein expression.

A peptide competition assay was performed using a synthetic LAG-3 peptide to further investigate the specificity of the LAG-3 IHC assay. The percentage of LAG-3–positive ICs in melanoma tissue was found to decrease from a starting staining level of 40% to <1% following preincubation with increasing molar ratios of a LAG-3 peptide (online supplemental table 4), indicating that the LAG-3 peptide bound competitively to the 17B4 clone.

To determine the range of LAG-3 IC expression in melanoma specimens, 100 commercially procured melanoma samples were assessed using the LAG-3 IHC assay. Of these 100 samples, 38 were positive for LAG-3 IC expression and 62 were negative, using 1% expression as a cut-off value (figure 2). The range of IC expression in the positive specimens was 1%–40%, with a median of 3%. Of the positive cases, the majority (36) had a LAG-3 IC staining intensity of 2+, 1 sample had a LAG-3 IC staining intensity of 3+ and 1 sample had a LAG-3 IC staining intensity of 1+. Taken together, these data indicate that the LAG-3 IHC assay detects varying levels of immune infiltrates expressing LAG-3 in human FFPE melanoma samples. Figure 3 shows representative tissue examples of staining from 0% to 30%.

Analytical precision of the LAG-3 IHC assay within the same laboratory

Twenty-four FFPE melanoma samples and one normal human tonsil tissue control sample were stained on two different Leica BOND-III instruments and subsequently scored by two independent pathologists to establish the repeatability and reproducibility of the LAG-3 IHC assay. The intrarun repeatability, interday, interinstrument, interoperator and inter-reagent lot reproducibility all demonstrated a high concordance, with all point estimates >95% in average negative agreement (ANA), average positive agreement (APA) and overall percentage agreement (OPA) (table 1).

Interobserver and intraobserver reproducibility of the LAG-3 IHC assay within the same laboratory

Evaluations of 60 melanoma samples performed by 3 independent pathologists from the same laboratory and repeat evaluations of the same 60 melanoma samples by the same pathologist were examined to determine the interobserver and intraobserver reproducibility of the assay within the same laboratory. To determine the interobserver reproducibility of the LAG-3 IHC assay, pairwise comparisons were made of the 180 diagnostic calls by the 3 pathologists: 91 were concordant for positive-to-positive calls, and 77 were concordant for negative-to-negative calls. Disagreements occurred in 12 cases, all of which had LAG-3 scores around the 1% threshold (LAG-3–positive IC content of 0%–1%), resulting in a lower point estimate and lower bound 95% CI for ANA compared with APA and OPA. Point estimates for ANA, APA and OPA were >90% with the lower bound 95% CIs >85% (table 2).

To determine intraobserver reproducibility of the LAG-3 IHC assay, the 60 samples assessed in the interobserver reproducibility testing were reassessed by the same pathologists, following a wash-out period. Among the 180 comparisons of diagnostic calls between 2 reads by 3 pathologists, 89 were positive-to-positive concordant, 78 were negative-to-negative concordant, 8 were negative-to-positive discordant and 5 were positive-to-negative discordant. Additionally, the point estimates and lower bound 95% CIs were >90% and >85%, respectively, in ANA, APA and OPA (table 2).

Interlaboratory and intralaboratory reproducibility of the LAG-3 IHC assay

Two experiments were performed to assess interlaboratory reproducibility: interobserver and intraobserver reproducibility, and overall interlaboratory and intralaboratory reproducibility. First, to investigate the interobserver and intraobserver reproducibility of the LAG-3 IHC assay between different laboratories, 70 melanoma LAG-3–prestained cases were assessed by 3 pathologists at 3 separate laboratories. Second, to determine overall interlaboratory and intralaboratory reproducibility, unstained slides from 24 melanoma cases that had previously been shown to have a range of LAG-3 expression were tested at 3 separate laboratories. The interobserver and intraobserver reproducibility and overall interlaboratory and intralaboratory reproducibility demonstrated assay staining and scoring concordance with point estimates for all studies at >90% in ANA, APA and OPA and lower bound 95% CIs >85% (table 3).

Slide stability experiments

To establish the stability of LAG-3 protein in unstained FFPE tissue sections on glass slides for the LAG-3 IHC assay, the concordance of sectioned tissue samples stained after different storage periods was measured. There was 100% concordance in scoring (positive or negative) at all timepoints for slides stored at ambient temperatures or 2°C–8°C. The LAG-3–positive IC staining intensity results for the tonsil tissue were 100% concordant from baseline through month 18 at both 2°C–8°C and ambient temperatures, with a decrease in LAG-3 IC staining intensity from 3+ to 2+ at month 24. Although there was some slight variation (increase or decrease) in the percentage
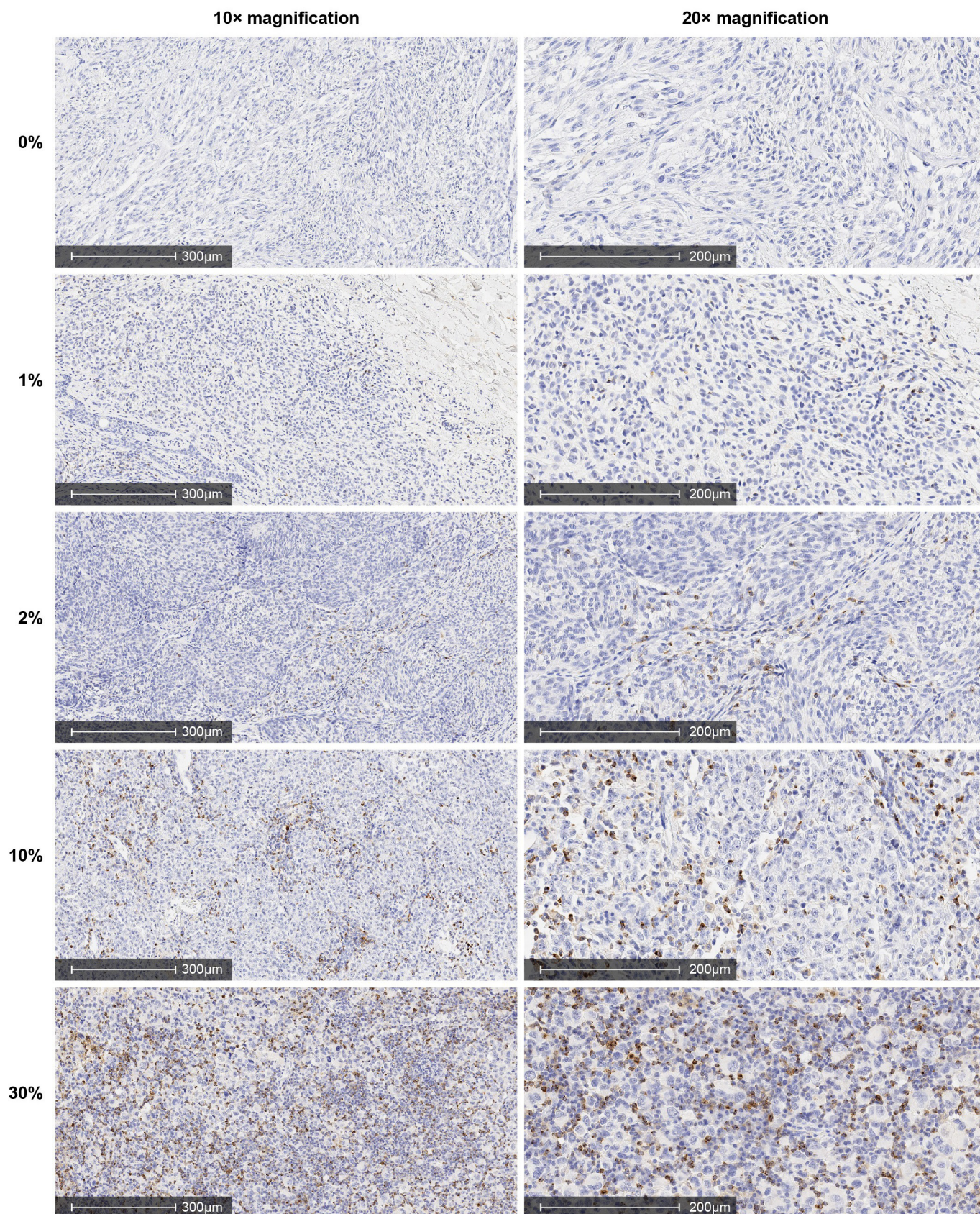
Figure 3 Examples of a range of LAG-3 expression levels detected in melanoma tissues using the LAG-3 IHC assay. Melanoma tissues showing a range of staining (0%–30%) for LAG-3 examined at magnifications of ×10 (left-hand image) and ×20 (right-hand image). IHC, immunohistochemistry; LAG-3, lymphocyte-activation gene 3.

Table 1 Summary of precision study results

Evaluation	Percentage agreement (95% CI)
Intrarun repeatability	ANA: 98.5 (97.3 to 99.6)
	APA: 98.6 (97.4 to 99.6)
	OPA: 98.5 (97.3 to 99.6)
Interday reproducibility	ANA: 97.4 (96.4 to 98.4)
	APA: 97.6 (96.6 to 98.5)
	OPA: 97.5 (96.5 to 98.4)
Interinstrument reproducibility	ANA: 97.8 (96.8 to 98.6)
	APA: 97.9 (97.0 to 98.7)
	OPA: 97.8 (97.0 to 98.6)
Interoperator reproducibility	ANA: 97.8 (96.8 to 98.6)
	APA: 97.9 (97.0 to 98.7)
	OPA: 97.8 (96.9 to 98.7)
Inter-reagent lot reproducibility	ANA: 97.4 (96.6 to 98.2)
	APA: 97.6 (96.8 to 98.3)
	OPA: 97.5 (96.7 to 98.3)

ANA, average negative agreement; APA, average positive agreement; OPA, overall percentage agreement.

Table 3 Percentage agreement and 95% CIs in the interlaboratory reproducibility study

Evaluation	Percentage agreement (95% CI)
Intraobserver reproducibility	ANA: 92.1 (89.6 to 94.4)
	APA: 94.2 (92.4 to 95.9)
	OPA: 93.3 (91.3 to 95.2)
Interobserver reproducibility	ANA: 90.2 (88.7 to 91.7)
	APA: 92.9 (91.7 to 94.0)
	OPA: 91.8 (90.5 to 93.0)
Intralaboratory reproducibility	ANA: 95.1 (93.3 to 96.7)
	APA: 96.0 (94.5 to 97.3)
	OPA: 95.6 (94.0 to 97.1)
Interlaboratory reproducibility	ANA: 93.2 (91.9 to 94.4)
	APA: 94.4 (93.4 to 95.5)
	OPA: 93.9 (92.7 to 94.9)

ANA, average negative agreement; APA, average positive agreement; OPA, overall percentage agreement.

of LAG-3–positive ICs for some melanoma samples during the course of testing (eg, a case reported as 2% at week 2, 1% at week 4 and 2% at month 2), the LAG-3 score (positive or negative) and LAG-3–positive IC staining intensity (1+, 2+, 3+) results were 100% concordant for individual samples tested at each timepoint and each temperature. The small differences observed may be attributable to variations in the density of ICs between tissue sections.

DISCUSSION

LAG-3 is a key immune checkpoint currently being investigated as an I-O therapy for patients with solid tumours and haematological malignancies.[13 16 18 21 26–28] The development of a robust LAG-3 IHC assay will enable the analysis of IC LAG-3 status in the tumour microenvironment and the correlation between LAG-3 expression status and response to LAG-3–directed oncology treatments. A robust LAG-3 IHC assay that is suitable for clinical trials and clinical use for melanoma is described in this work. The specificity of the assay was demonstrated using cell lines with *LAG3* gene disruptions and with a peptide antigen competition assay. LAG-3 scoring was reported as the percentage of LAG-3–positive ICs (which morphologically resembled lymphocytes) relative to all nucleated cells within the overall tumour region. A ≥1% cut-off was used to determine LAG-3 positivity. Analytical precision was demonstrated for intrarun repeatability, interday, interinstrument, interoperator and inter-reagent lot reproducibility, with concordance >95%. Pathologist

Table 2 Percentage agreement and 95% CIs for interobserver and intraobserver agreement within the same laboratory

Evaluation	Percentage agreement (95% CI)
Interobserver reproducibility	ANA: 92.8 (88.31 to 96.59)
	APA: 93.8 (89.95 to 97.06)
	OPA: 93.3 (89.44 to 96.66)
Intraobserver reproducibility	ANA: 92.31 (87.74 to 96.09)
	APA: 93.19 (89.22 to 96.52)
	OPA: 92.78 (88.89 to 96.11)

ANA, average negative agreement; APA, average positive agreement; OPA, overall percentage agreement.

interobserver and intraobserver reproducibility was >90% in terms of ANA, APA and OPA. LAG-3 was observed to be stable in unstained tissues mounted on glass slides, with concordant staining observed in samples stored at both 2°C–8°C and ambient temperatures for up to 24 months. These data demonstrate that this assay can reproducibly determine the proportion of LAG-3–positive ICs within a sample. Despite challenges associated with the scoring of ICs, the LAG-3 IHC assay demonstrated a high level of interobserver reproducibility both within the same laboratory and between independent laboratories.[29 30]

A particular issue for the interpretation of IHC assays for melanoma tissues is the presence of melanin pigment. Melanin pigmentation can interfere with IHC interpretation, as it may obscure morphological features and is similar in colour to the chromogen 3,3'-diaminobenzidine tetrahydrochloride hydrate, which is commonly used in IHC assays, including the LAG-3 IHC assay described here. The pretreatment method described in this work removed melanin from samples without compromising the LAG-3 antigen and resulted in no samples that could not be interpreted due to excess melanin pigmentation.

One limitation of the studies presented in this work is that a number of preanalytical factors may impact the performance of the LAG-3 IHC assay, including location of the tissue assessed (ie, primary vs metastatic),[31 32] sample ischemia time and fixation methods.[33] Additionally, the design of the cut slide stability studies compared LAG-3 staining and IC expression with baseline (time 0), but did not include comparison with other timepoints.

The assay described in this report was used to stratify patients based on LAG-3 expression in RELATIVITY-047 (NCT03470922), a phase II/III clinical trial in patients with previously untreated metastatic or unresectable melanoma. The trial compared combined nivolumab (anti–PD-1) and relatlimab (anti–LAG-3) treatment with nivolumab monotherapy, and benefit of combination therapy was observed in comparison with nivolumab monotherapy.[21] While the median PFS estimates were longer for patients with LAG-3 expression ≥1% across both treatment groups, a benefit with the combination therapy over nivolumab was observed regardless of LAG-3 expression.[21] As described above, the FDA recently approved nivolumab and relatlimab-rmbw (Opdualag; relatlimab-rmbw is the name used when referring to the approval by FDA: as per the agency naming guidance, the naming convention for biological products licensed under the Public Health Service Act is a proper name

consisting of a core name and an FDA-designated suffix.[22]) for adult and paediatric patients ≥12 years of age with unresectable or metastatic melanoma.[23] Opdualag is a fixed-dose combination of the LAG-3–blocking antibody relatlimab and the anti–PD-1 antibody nivolumab.

Both the present report and RELATIVITY-047 determined LAG-3 positivity using a ≥1% cut-off.[21] However, the prevalence of LAG-3 positivity observed in other sample sets or patient populations may vary, meaning cut-off values for clinical utility will have to be determined and validated in clinical studies. For instance, Dillon *et al* reported a higher prevalence of LAG-3 positivity using a ≥1% cut-off in a different set of commercially procured FFPE melanoma samples than in the melanoma samples used in this report.[34] Dillon *et al* also reported a higher prevalence of LAG-3 positivity in gastric and gastrooesophageal cancer samples than in the melanoma samples used in this report. The LAG-3 assay described in this manuscript is currently being utilised in a number of clinical trials for multiple different tumour types.

In summary, a robust IHC assay for the determination of LAG-3 IC status in the tumour microenvironment in solid tumour tissues has been developed.

Take home messages

⇒ Lymphocyte-activation gene 3 (LAG-3) is an immune checkpoint receptor expressed on immune cells (ICs) that limits T-cell activity and is being actively explored as a target for immunotherapy.

⇒ An immunohistochemistry (IHC) assay was developed to detect the LAG-3 protein in formalin-fixed paraffin-embedded human tumour tissue specimens. This study describes scoring criteria and shows the specificity, sensitivity, analytical precision and reproducibility of this assay as an aid to determine LAG-3 expression in melanoma patients using a ≥1% expression on ICs threshold.

⇒ The study describes a key immuno-oncology checkpoint IHC assay that is robust and suitable for clinical trials. The assay was used in RELATIVITY-047 (NCT03470922), a phase II/III clinical trial that compared combined nivolumab and relatlimab treatment with nivolumab monotherapy, to stratify patients based on the percentage of LAG-3–positive ICs within the tumour region. This assay is also being used in several ongoing clinical trials evaluating clinical response to relatlimab.

ORCID iD
Jeffrey Shuster http://orcid.org/0000-0002-3820-1743

REFERENCES

1. Vaddepally RK, Kharel P, Pandey R, *et al*. Review of indications of FDA-approved immune checkpoint inhibitors per NCCN guidelines with the level of evidence. *Cancers* 2020;12:738.
2. Guo L, Wei R, Lin Y, *et al*. Clinical and recent patents applications of PD-1/PD-L1 targeting immunotherapy in cancer treatment-current progress, strategy, and future perspective. *Front Immunol* 2020;11:1508.
3. Larkin J, Chiarion-Sileni V, Gonzalez R, *et al*. Combined nivolumab and ipilimumab or monotherapy in untreated melanoma. *N Engl J Med* 2015;373:23–34.
4. Ascierto PA, Del Vecchio M, Mandalá M, *et al*. Adjuvant nivolumab versus ipilimumab in resected stage IIIB-C and stage IV melanoma (CheckMate 238): 4-year results from a multicentre, double-blind, randomised, controlled, phase 3 trial. *Lancet Oncol* 2020;21:1465–77.
5. Eggermont AMM, Blank CU, Mandalà M, *et al*. Adjuvant pembrolizumab versus placebo in resected stage III melanoma (EORTC 1325-MG/KEYNOTE-054): distant metastasis-free survival results from a double-blind, randomised, controlled, phase 3 trial. *Lancet Oncol* 2021;22:643–54.
6. Hellmann MD, Paz-Ares L, Bernabe Caro R, *et al*. Nivolumab plus ipilimumab in advanced non-small-cell lung cancer. *N Engl J Med* 2019;381:2020–31.
7. Paz-Ares L, Ciuleanu T-E, Cobo M, *et al*. First-line nivolumab plus ipilimumab combined with two cycles of chemotherapy in patients with non-small-cell lung cancer (CheckMate 9LA): an international, randomised, open-label, phase 3 trial. *Lancet Oncol* 2021;22:198–211.
8. Ferris RL, Blumenschein G, Fayette J, *et al*. Nivolumab for recurrent squamous-cell carcinoma of the head and neck. *N Engl J Med* 2016;375:1856–67.
9. Burtness B, Harrington KJ, Greil R, *et al*. Pembrolizumab alone or with chemotherapy versus cetuximab with chemotherapy for recurrent or metastatic squamous cell carcinoma of the head and neck (KEYNOTE-048): a randomised, open-label, phase 3 study. *Lancet* 2019;394:1915–28.
10. Bellmunt J, de Wit R, Vaughn DJ, *et al*. Pembrolizumab as second-line therapy for advanced urothelial carcinoma. *N Engl J Med* 2017;376:1015–26.
11. Bajorin DF, Witjes JA, Gschwend J, *et al*. First results from the phase 3 CheckMate 274 trial of adjuvant nivolumab vs placebo in patients who underwent radical surgery for high-risk muscle-invasive urothelial carcinoma (MIUC). *JCO* 2021;39(suppl 6):391.

12 Huang R-Y, Francois A, McGray AR, *et al*. Compensatory upregulation of PD-1, LAG-3, and CTLA-4 limits the efficacy of single-agent checkpoint blockade in metastatic ovarian cancer. *Oncoimmunology* 2017;6:e1249561.

13 Long L, Zhang X, Chen F, *et al*. The promising immune checkpoint LAG-3: from tumor microenvironment to cancer immunotherapy. *Genes Cancer* 2018;9:176–89.

14 Camisaschi C, Casati C, Rini F, *et al*. LAG-3 expression defines a subset of CD4(+) CD25(high)Foxp3(+) regulatory T cells that are expanded at tumor sites. *J Immunol* 2010;184:6545–51.

15 Grosso JF, Kelleher CC, Harris TJ, *et al*. LAG-3 regulates CD8+ T cell accumulation and effector function in murine self- and tumor-tolerance systems. *J Clin Invest* 2007;117:3383–92.

16 Woo S-R, Turnis ME, Goldberg MV, *et al*. Immune inhibitory molecules LAG-3 and PD-1 synergistically regulate T-cell function to promote tumoral immune escape. *Cancer Res* 2012;72:917–27.

17 Keane C, Law SC, Gould C, *et al*. LAG3: a novel immune checkpoint expressed by multiple lymphocyte subsets in diffuse large B-cell lymphoma. *Blood Adv* 2020;4:1367–77.

18 Workman CJ, Cauley LS, Kim I-J, *et al*. Lymphocyte activation gene-3 (CD223) regulates the size of the expanding T cell population following antigen activation in vivo. *J Immunol* 2004;172:5450–5.

19 Wang J, Sanmamed MF, Datar I, *et al*. Fibrinogen-like protein 1 is a major immune inhibitory ligand of LAG-3. *Cell* 2019;176:334–47. e12.

20 Matsuzaki J, Gnjatic S, Mhawech-Fauceglia P, *et al*. Tumor-infiltrating NY-ESO-1-specific CD8+ T cells are negatively regulated by LAG-3 and PD-1 in human ovarian cancer. *Proc Natl Acad Sci U S A* 2010;107:7875–80.

21 Tawbi HA, Schadendorf D, Lipson EJ, *et al*. Relatlimab and nivolumab versus nivolumab in untreated advanced melanoma. *N Engl J Med* 2022;386:24–34.

22 US Food and Drug Administration. Nonproprietary naming of biological products, 2017. Available: https://www.fda.gov/files/drugs/published/Nonproprietary-Naming-of-Biological-Products-Guidance-for-Industry.pdf [Accessed 04 Apr 2022].

23 Bristol Myers Squibb. OPDUALAGTM (relatlimab) [package insert], 2022. Available: https://www.accessdata.fda.gov/drugsatfda_docs/label/2022/761234s000lbl.pdf [Accessed 04 Apr 2022].

24 Baixeras E, Huard B, Miossec C, *et al*. Characterization of the lymphocyte activation gene 3-encoded protein. A new ligand for human leukocyte antigen class II antigens. *J Exp Med* 1992;176:327–37.

25 Woo S-R, Li N, Bruno TC, *et al*. Differential subcellular localization of the regulatory T-cell protein LAG-3 and the coreceptor CD4. *Eur J Immunol* 2010;40:1768–77.

26 ClinicalTrials.gov. A study to assess adjuvant immunotherapy with Relatlimab and nivolumab versus nivolumab alone after complete resection of stage III-IV melanoma (RELATIVITY-098), 2022. Available: https://clinicaltrials.gov/ct2/show/NCT05002569 [Accessed 11 Feb].

27 ClinicalTrials.gov. A study to evaluate the safety, tolerability, and efficacy of Relatlimab in relapsed or refractory B-cell malignancies, 2022. Available: https://clinicaltrials.gov/ct2/show/NCT02061761 [Accessed 11 Feb].

28 Morgensztern D, Chaudhry A, Iannotti N, Acevedo A, *et al*. 1359TiP RELATIVITY-104: first-line relatlimab (RELA) + nivolumab (NIVO) with chemotherapy vs nivo with chemotherapy in stage IV or recurrent non-small cell lung cancer (NSCLC): a phase II, randomized, double-blind study. *Ann Oncol* 2021;32:S1030.

29 Adam J, Le Stang N, Rouquette I, *et al*. Multicenter harmonization study for PD-L1 IHC testing in non-small-cell lung cancer. *Ann Oncol* 2018;29:953–8.

30 Rimm DL, Han G, Taube JM, *et al*. A prospective, multi-institutional, pathologist-based assessment of 4 immunohistochemistry assays for PD-L1 expression in non-small cell lung cancer. *JAMA Oncol* 2017;3:1051–8.

31 Peng L, Zhang Z, Zhao D, *et al*. Discordance of immunohistochemical markers between primary and recurrent or metastatic breast cancer: a retrospective analysis of 107 cases. *Medicine* 2020;99:e20738.

32 Rozenblit M, Huang R, Danziger N, *et al*. Comparison of PD-L1 protein expression between primary tumors and metastatic lesions in triple negative breast cancers. *J Immunother Cancer* 2020;8:e001558.

33 Ramos-Vara JA, Miller MA. When tissue antigens and antibodies get along: revisiting the technical aspects of immunohistochemistry--the red, brown, and blue technique. *Vet Pathol* 2014;51:42–87.

34 Dillon LM, Wojcik J, Desai K. Abstract 1625: distribution and prevalence of LAG-3 expression in samples of melanoma and gastric/gastroesophageal junction cancer. *Cancer Res* 2021;81(suppl 13)–1625–25.

Supplemental material

BMJ Publishing Group Limited (BMJ) disclaims all liability and responsibility arising from any reliance placed on this supplemental material which has been supplied by the author(s)

J Clin Pathol

SUPPLEMENTAL MATERIAL

SUPPLEMENTAL METHODS

Staining procedures

Formalin-fixed, paraffin-embedded (FFPE) tissue sections were deparaffinized by immersing slides through xylene and graded alcohols prior to staining. Variable amounts of melanin pigment are typically found in melanoma tumors, potentially impacting the interpretation of the lymphocyte-activation gene 3 (LAG-3) immunohistochemistry (IHC) assay. To attenuate melanin pigment in the melanoma samples, deparaffinized slides were placed in melanin-removal agent (1 part Dako Target Retrieval solution pH 9, 10× concentrate [Agilent, Cat. # S236784-2], 8 parts methanol with 1 part hydrogen peroxide, 30% w/w, added) and incubated in a Decloaking Chamber™ NxGen (BioCare Medical, Pacheco, CA; part no. DC2012) for 3 hours at 60°C, then rinsed in deionized water.

Following melanin removal ("bleaching"), the LAG-3 IHC assay was performed in the BOND-III automated staining system (Leica Biosystems) as follows (summarized in **supplemental Table 1**):

1. Antigen unmasking of the FFPE tissue sections was done by incubating samples in BOND™ Epitope Retrieval Solution 1 (Leica Biosystems, Cat. # AR9961) for 20 minutes at 100°C

2. Endogenous peroxidase activity was quenched by incubation in pre-primary peroxidase inhibitor (BOND™ Polymer Refine Detection; Leica Biosystems, Cat. # DS9800) for 5 minutes at ambient temperature (20–25°C)

3. Slides were incubated with a protein block (Dako Serum Free Protein Block; Agilent, Cat. # X090930-2) for 5 minutes and then incubated with the LAG-3 primary antibody, diluted to 2.5 µg/mL (1:400) in BOND Primary Antibody Diluent (Leica Biosystems, Cat. # AR9352) or negative control antibody (mouse monoclonal immunoglobulin G1 [IgG1]; clone MOPC-21; Leica Biosystems, Cat. # PA0996) for 30 minutes at ambient temperature

4. The primary antibody was washed off and the slides incubated with the post-primary rabbit anti-mouse immunoglobulin G (IgG) linker reagent (BOND™ Polymer Refine Detection; Leica Biosystems, Cat. # DS9800) for 8 minutes at ambient temperature

5. Incubation with the secondary polymer anti-rabbit poly–horseradish peroxidase-IgG was done for 8 minutes, followed by incubation with the 3,3'-diaminobenzidine tetrahydrochloride hydrate (DAB) chromogen (BOND™ Polymer Refine Detection; Leica Biosystems, Cat. # DS9800) for 10 minutes at ambient temperature

6. After washing off the excess DAB, sample nuclei were counterstained with hematoxylin (BOND™ Polymer Refine Detection; Leica Biosystems, Cat. # DS9800) for 5 minutes at ambient temperature

Melanoma tissue staining was performed with 3 run controls: melanoma biopsy tissue with LAG-3 immune cell (IC) expression >5% (predetermined by IHC) was used as a

2

Supplemental material

BMJ Publishing Group Limited (BMJ) disclaims all liability and responsibility arising from any reliance placed on this supplemental material which has been supplied by the author(s)

J Clin Pathol

positive control, tonsil tissue with areas differentiated by positive or negative LAG-3 IC expression was used as a positive and negative control, and nonimmune mouse IgG was used as a negative reagent control. Slides were reviewed by a pathologist using bright field microscopy. If either the positive melanoma tissue control or the tonsil tissue control was deemed unacceptable by the interpreting pathologist, the staining run was repeated. To be considered acceptable, the positive melanoma tissue control must have had a LAG-3 IC expression score (see **LAG-3 scoring** section) of >5%, and the tonsil tissue must have had positive staining on ICs in germinal centers or interfollicular regions with no staining within the crypt epithelium, skeletal and smooth muscle fibers, collagen fibers, adipose tissue, and peripheral nerves.

Generation of CRISPR-engineered LAG-3 knockout cell lines

Pooled clustered regularly interspaced short palindromic repeats (CRISPR)-engineered COV434 ovarian granulosa tumor cells (TCs) with heterogeneous LAG-3 expression were derived using 3 unique, nonoverlapping CRISPR guides targeting different regions of exon 2 of LAG-3. Guide sequences were: Cr1, TGACCCCTGCTCTTCGCAGA; Cr2, GATCCTGGAGGGGGATTGTG; Cr3, GCCAGGGGCTGAGGTCCCGG. Editing frequency was assessed by next-generation sequencing. In total, 3 cell line pools were derived with different frequencies of modification, which led to absence of LAG-3 protein expression but not LAG-3 mRNA expression.

Peptide inhibition assay

A peptide corresponding to the immunogen used to generate antibody 17B4, GPPAAAPGHPLAPGPHPAAPSSWGPRPRRY, was synthesized and combined in various molar ratios (0-fold, 1-fold, 2-fold, 5-fold, 10-fold, and 30-fold excess) with

3

Supplemental material

BMJ Publishing Group Limited (BMJ) disclaims all liability and responsibility arising from any reliance placed on this supplemental material which has been supplied by the author(s)

J Clin Pathol

antibody 17B4 in phosphate buffer saline solution for 30 minutes at ambient temperature and centrifuged at 16,000 × g at 4°C for 15 minutes. Supernatants from centrifuged aliquots were used as the primary antibody solution in the LAG-3 IHC assay performed on FFPE melanoma tissue previously scored with >5% LAG-3–positive ICs.

Precision Study Measurements

The agreements of LAG-3 scores were assessed to determine the intrarun repeatability and interday, interinstrument, interoperator, and interreagent lot reproducibility. Samples for this study consisted of 1 normal human tonsil to serve as both a positive and negative control and 24 FFPE melanoma tissues previously confirmed to have a range of LAG-3 IC expression (12 were LAG-3–positive [≥1%] with a range of 1%–40%, and 12 were LAG-3–negative [<1%]). Slides were sectioned from each FFPE melanoma tissue block as described above. One slide from each of the 24 melanoma tissue blocks was stained for hematoxylin and eosin (H&E). Each sample was tested on 5 nonconsecutive days, with 2 independent runs by 2 different operators on each day following a wash-out period.

Intrarun Repeatability

Intrarun duplicates were included in 2 independent runs per sample each day. All slides were evaluated by 2 pathologists. Each pathologist had 240 intrarun duplicates (24 specimens, ran twice each day for 5 days) evaluated for agreement, and 2 pathologists had a total of 480 combined pairwise comparisons to compute average negative agreement (ANA), average positive agreement (APA), and overall percentage agreement (OPA).

4

Interday Reproducibility

Two independent runs were performed each day. Each sample was run in duplicate and evaluated by 2 different pathologists who consolidated their evaluations into 1 call representing the run (agreed or discordant). For each pathologist or run, there were 10 interday pairwise comparisons per specimen (5 × 2). For both pathologists and runs, there were 20 interday pairwise comparisons (5 × 2 × 2). There were 480 (24 × 10 × 2) interday pairwise comparisons from 1 pathologist and 960 interday pairwise comparisons from 2 pathologists' reads to be evaluated for ANA, APA, and OPA.

Interinstrument Reproducibility

Two different Leica BOND-III instruments were used by each operator each day during the interday reproducibility testing; due to the limited number of slides allowed on each instrument, 12 specimens were run on 1 instrument while the other 12 were run on the other instrument. Because of the design and rotation of 2 instruments in 5 testing days, the number of runs on 2 instruments for 1 specimen were 6 and 4. The number of total interinstrument pairwise comparisons were 24 per specimen (6 × 4), 576 for all 24 specimens (24 × 24) for 1 pathologist, and 1152 (2 × 576) to be evaluated in total for 2 pathologists' reads. The total 1152 pairwise interinstrument comparisons were used to compute ANA, APA, and OPA.

Interoperator Reproducibility

Each sample was evaluated in 2 independent runs by 2 different operators each day. The number of runs by each operator was 5 for each sample. The total number of pairwise comparisons between the 2 operators per sample was 25 (5 × 5) and 600 (24 × 25) pairwise comparisons for all 24 specimens for each pathologist's read. In total,

5

Supplemental material

BMJ Publishing Group Limited (BMJ) disclaims all liability and responsibility arising from any reliance placed on this supplemental material which has been supplied by the author(s)

J Clin Pathol

there were 1200 interoperator pairwise comparisons for 2 pathologists. The 1200 pairwise comparisons were used to compute ANA, APA, and OPA.

Interreagent Lot Reproducibility

Three reagent lots were used in rotation of 2 lots for each of the 5 testing days during the interday reproducibility testing, resulting in 4 runs with the first 2 lots and 2 runs with the third lot. The interreagent lot pairwise comparisons were 32 per specimen ([4 × 4] + [4 × 2] + [4 × 2]), adding up to 768 for all 24 specimens (32 × 24) for each pathologist's evaluation. In total, 1536 (2 × 768) interlot pairwise comparisons were used to compute ANA, APA, and OPA.

Reproducibility Within the Same Laboratory

Interobserver Reproducibility

Sixty melanoma samples with a range of staining intensity and a minimum of 15% of challenging cases around the prespecified threshold (≥1%) were assessed by 3 independent, board-certified anatomic pathologists from the same laboratory. Samples were randomized and blinded prior to evaluation. Three pairwise comparisons were made and pooled to estimate ANA, APA, and OPA.

Intraobserver Reproducibility

The same 60 samples used to determine interobserver reproducibility were re-evaluated by the same 3 pathologists, following a wash-out period between original evaluations and re-evaluations. Results from the re-evaluations were compared with the original

6

Supplemental material

BMJ Publishing Group Limited (BMJ) disclaims all liability and responsibility arising from any reliance placed on this supplemental material which has been supplied by the author(s)

J Clin Pathol

evaluations to assess intraobserver precision. Pathologists were blinded to original results, and slides were re-randomized prior to examination. Three intraobserver pairwise comparisons were made for each of the 3 pathologists and pooled to provide average intraobserver agreement from all 3 pathologists.

Reproducibility Across Independent Laboratories

Interobserver and Intraobserver Reproducibility in Different Laboratories

Seventy melanoma samples with a range of staining intensity were assessed by 3 pathologists from 3 separate laboratories. Assessment occurred over 3 days at least 14 days apart, with 210 reads per pathologist. For intraobserver reproducibility, ANA, APA, and OPA were computed using all non-redundant pairwise comparisons for a single observer. For interobserver reproducibility, all non-redundant pairwise comparisons between pathologists (including laboratory 1 vs. laboratory 2, laboratory 1 vs. laboratory 3, and laboratory 2 vs. laboratory 3) were used to compute ANA, APA, and OPA.

Interlaboratory and Intralaboratory Reproducibility

Twenty-four melanoma cases with a range of LAG-3 IHC expression were tested on 5 different days at each of the 3 different laboratories. Intralaboratory ANA, APA, and OPA were computed using a pool of all possible nonredundant pairwise intralaboratory comparisons. Interlaboratory ANA, APA, and OPA were calculated using a pool of all possible nonredundant pairwise interlaboratory comparisons.

7

Statistical Methods

ANA, APA, and OPA were calculated for intrarun repeatability and interday, interinstrument, interreagent lot, interobserver and intraobserver, and interlaboratory and intralaboratory reproducibility measurements. 95% confidence intervals were calculated using the percentile bootstrap method.[1]

Stability Experiments in FFPE Sections

Slides were sectioned from 6 melanoma FFPE tissue blocks spanning the dynamic range of LAG-3 expression and 1 tonsil FFPE tissue block as described in the ***Tissue Specimens*** section. Half of the slides were stored at ambient temperature, and half were stored at 2–8°C. Two of the slides stored at ambient temperature, and 2 of the slides stored at 2–8°C were used for testing at different time periods: at time 0 (baseline), at 1, 2, and 4 weeks, and then at 2, 3, 4, 5, 6, 8, 10, 12, 14, 18, and 24 months. Using the LAG-3 IHC assay, 1 slide was stained with LAG-3 antibody and 1 slide with nonimmune mouse IgG. A tonsil tissue control was included with each staining run as a positive and negative control, as described in the **Staining Procedures** section.

8

SUPPLEMENTAL TABLE 1. Summary of the LAG-3 IHC Assay Staining Procedure Steps.

Step	Procedure	Process	Reagent
1	Antigen retrieval	BOND™ Epitope Retrieval Solution 1, 20 min, 100°C	Leica Biosystems, Cat. # AR9961
2	Pre-primary peroxidase activity inhibition	BOND™ Polymer Refine Detection, 5 min, ambient temperature	Leica Biosystems, Cat. # DS9800
3	Protein block	Dako Serum Free Protein Block, 5 min	Agilent, Cat. # X090930-2
3	Primary antibody	Clone 17B4 (2.5 µg/mL) in BOND™ Primary Antibody Diluent or negative control antibody, 30 min, ambient temperature	Labcorp (antibody) Leica Biosystems (antibody diluent), Cat. # AR9352
4*	Post-primary rabbit anti-mouse IgG linker	BOND™ Polymer Refine Detection, <10 µg/mL in 10% (v/v) animal serum in TBS/0.1% ProClin™ 950, 8 min	Leica Biosystems, Cat. # DS9800

9

5	Polymer anti-rabbit poly-HRP-IgG	BOND™ Polymer Refine Detection, 8 min	Leica Biosystems, Cat. # DS9800
	DAB chromogen	BOND™ Polymer Refine Detection, 66 mM in stabilizer solution, 10 min	Leica Biosystems, Cat. # DS9800
6[†]	Hematoxylin counterstain	BOND™ Polymer Refine Detection, 5 min	Leica Biosystems, Cat. # DS9800

*Remove primary antibody by washing prior to this step.

[†]Remove excess DAB by washing prior to this step.

DAB, 3,3'-diaminobenzidine tetrahydrochloride hydrate; HRP, horse-radish peroxidase; IgG, immunoglobulin G; min, minutes; IHC, immunohistochemistry; LAG-3, lymphocyte-activation gene 3; TBS; tris-buffered saline; v/v, volume/volume.

10

SUPPLEMENTAL TABLE 2. Melanin Interpretation and Scoring Criteria.

Interpretation	Staining Description	LAG-3 IHC Assay Scoring Acceptability
0	No melanin pigment observed	Yes
1+	1 to 2 small foci in melanin containing tumor cells or macrophages	Yes
2+	More than 2 small foci of moderate to strong melanin or diffuse weak melanin with sufficient areas not obscured by melanin	Yes
3+	Diffuse weak to moderate melanin obscuring a significant portion of the tumor region	No
4+	Diffuse moderate to strong melanin obscuring most of the tumor region	No

IHC, immunohistochemistry; LAG-3, lymphocyte-activation gene 3.

11

SUPPLEMENTAL TABLE 3. LAG-3 Overall Stain Intensity Interpretation Criteria.

| Interpretation	Staining Description
1+	Weak LAG-3–positive IC staining: light brown or very punctate staining that may require high-power (40×) examination to detect
2+	Moderate LAG-3–positive IC staining: moderate to dark brown staining that is easily visible with 20× objective
3+	Strong LAG-3–positive IC staining: dark brown staining that is easily visible with 10× or 20× objective and obscures cell detail

IC, immune cells; LAG-3, lymphocyte-activation gene 3.

Supplemental material

BMJ Publishing Group Limited (BMJ) disclaims all liability and responsibility arising from any reliance placed on this supplemental material which has been supplied by the author(s)

J Clin Pathol

SUPPLEMENTAL TABLE 4. LAG-3 IHC peptide competition validation results.

Specimen (peptide:antibody ratio)	% LAG-3–positive ICs	Staining intensity
Melanoma LAG-3 mAb (0:1)	40	2+
Melanoma LAG-3 peptide (1:0)	0	N/A
Melanoma (1:1)	40	2+
Melanoma (2:1)	30	2+
Melanoma (5:1)	10–20	1–2+
Melanoma (10:1)	2	1+
Melanoma (30:1)	<1	1+

ICs, immune cells; IHC, immunohistochemistry; LAG-3, lymphocyte-activation gene 3;

mAb, monoclonal antibody; N/A, not applicable.

13

SUPPLEMENTAL FIGURE 1. LAG-3 IC scoring method overview. H&E, hematoxylin and eosin; IC, immune cells; LAG-3, lymphocyte-activation gene 3.
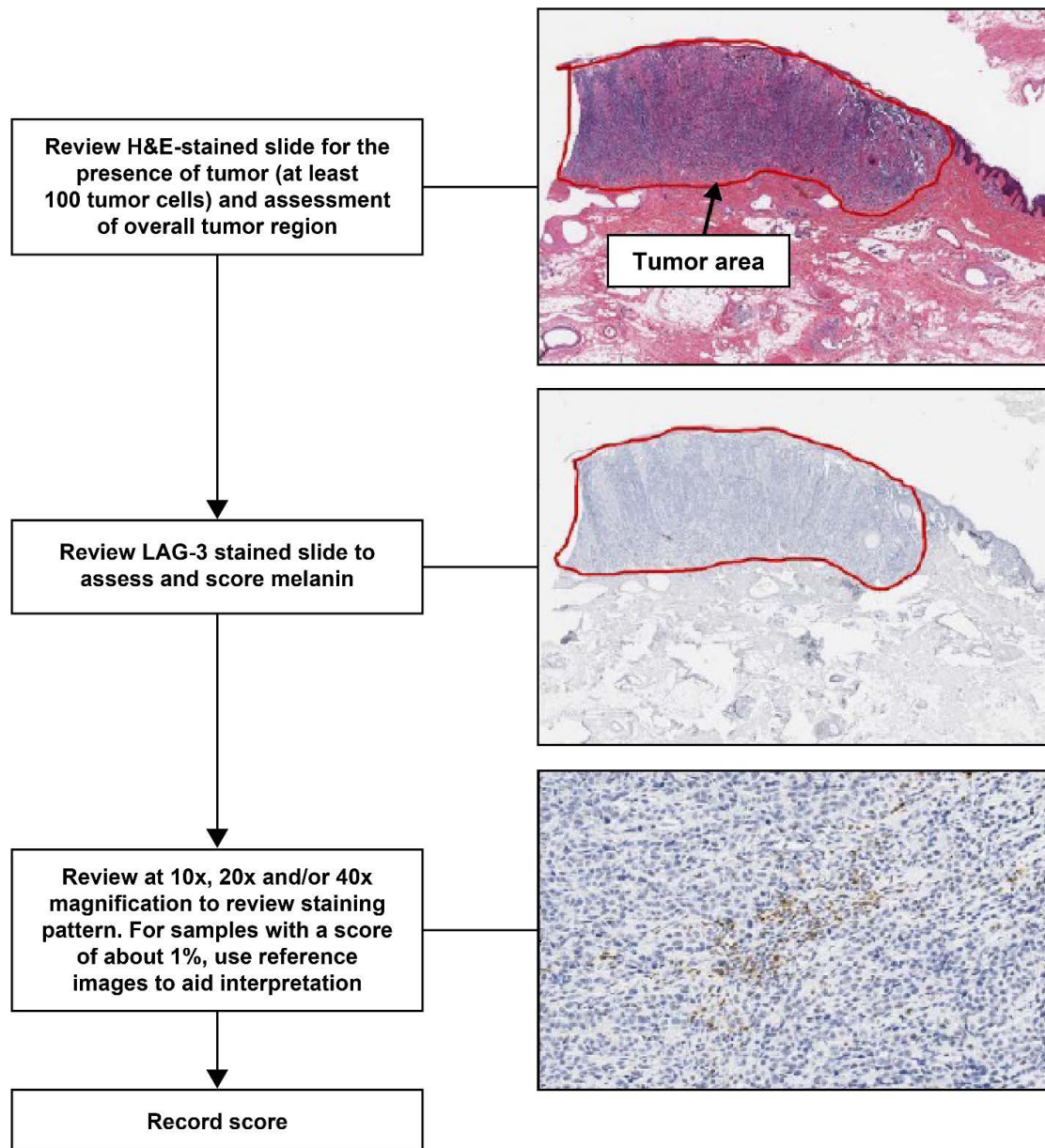
SUPPLEMENTAL FIGURE 2. Examples of punctate, membrane, and cytoplasmic LAG-3 IC staining observed with the LAG-3 IHC assay. Image shown at 40× magnification. IC, immune cells; IHC, immunohistochemistry; LAG-3, lymphocyte-activation gene 3.
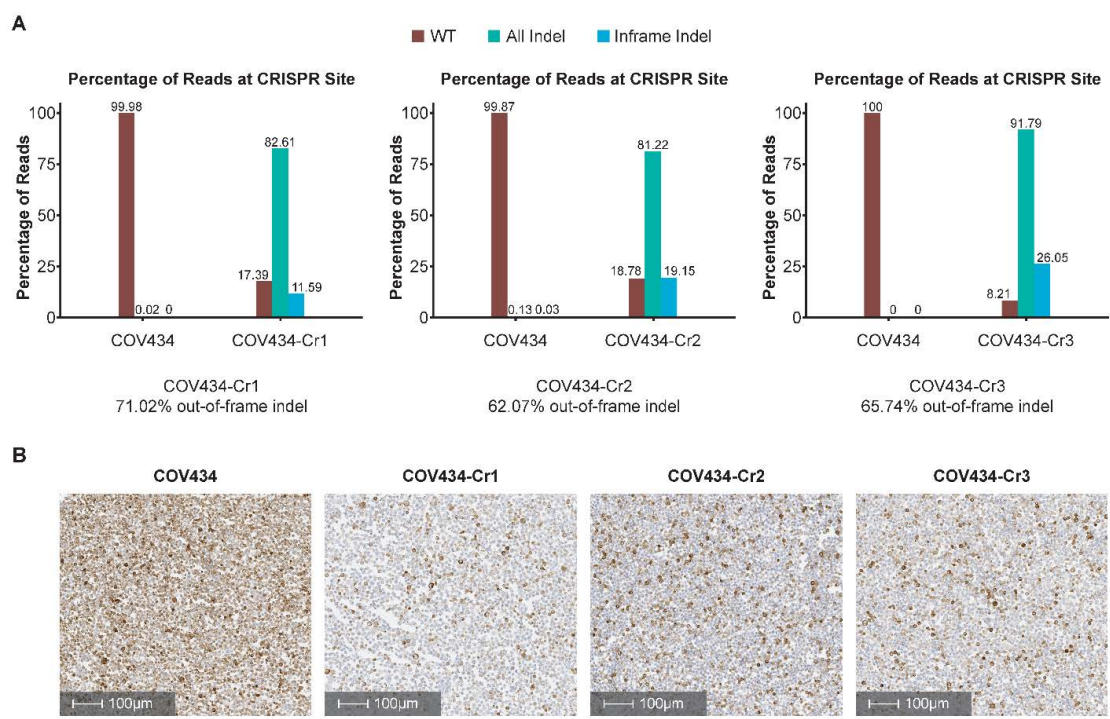

15

Supplemental material

BMJ Publishing Group Limited (BMJ) disclaims all liability and responsibility arising from any reliance placed on this supplemental material which has been supplied by the author(s)

J Clin Pathol
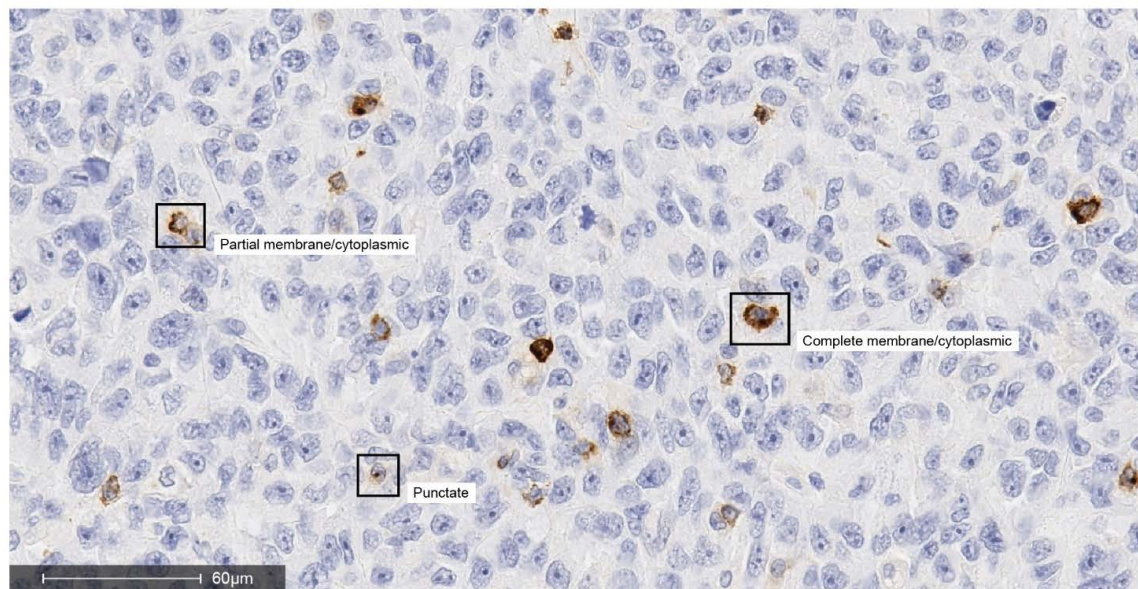
SUPPLEMENTAL FIGURE 3. Examples of the regions of a slide that were scored or not scored. A, Melanoma tissue with LAG-3–stained lymphocytes. The area scored includes the TC area and the PTS (outlined in red on the left panel). Adjacent normal (N) or uninvolved areas (shaded in pink) were not scored. Left panel image is shown at 10× magnification, right panel image is shown at 40× magnification. B, H&E–stained melanoma metastatic in lymph node. The area scored is shaded in blue (left panel) and included the TC area and PTS. Adjacent LN, shaded in pink (left panel), was not scored. The image is shown at 10× magnification. H&E, hematoxylin and eosin; LAG-3, lymphocyte-activation gene 3; LN, lymph node; PTS, peritumoral stroma; TC, tumor cell.
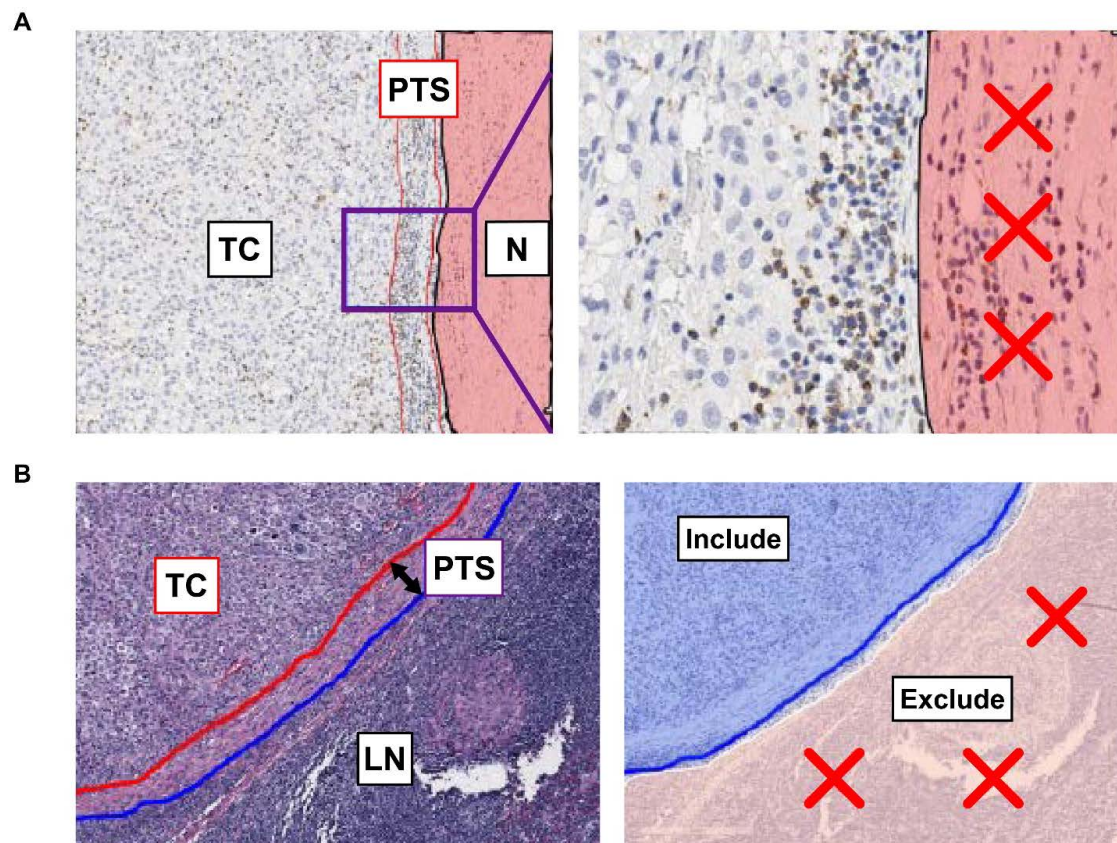
SUPPLEMENTAL FIGURE 4. Examples of LAG-3 IC staining in melanoma samples containing various levels of melanin pigment. A, Example of 1+ melanin pigmentation showing a single focus of tumor cells and macrophages with melanin. Image shown at 1× magnification (inset at 20×). B, Example of 2+ melanin pigmentation showing 2 large areas containing pigmented tumor cells and macrophages (outlined in red) showing weak to moderate melanin. Left-hand image shown at 2× magnification and right-hand image at 10×. C, Example of 3+ melanin pigmentation showing diffuse areas of moderately pigmented tumor cells and macrophages (outlined in red). An area with only limited melanin is present (outlined in green). Left-hand image shown at 0.7× magnification and right-hand image at 10×. D, Example of 4+ melanin pigmentation showing diffuse areas of strongly pigmented tumor cells and macrophages. LAG-3– stained lymphocytes cannot be visualized in the entire sample. Left-hand image is shown at 2× magnification and right-hand image at 10×. IC, immune cells; LAG-3, lymphocyte-activation gene 3.

17
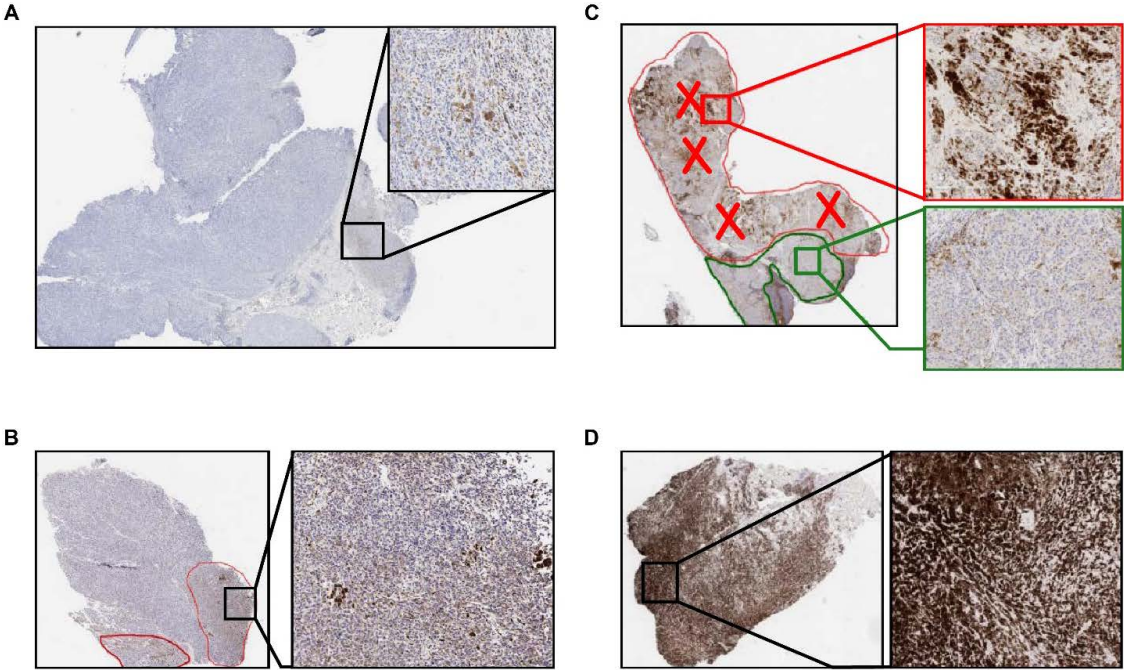
SUPPLEMENTAL FIGURE 5. Detection of LAG-3 expression in parental COV434 cells and LAG-3–disrupted COV434 cells. A, Bar charts showing NGS results from each of the pooled CRISPR-engineered COV434 cell lines. B, IHC staining showing LAG-3 expression in parental COV434 cells and the 3 pooled CRISPR-engineered COV434 cell lines. Tonsil tissue was used as a positive/negative control for the IHC staining. CRISPR, clustered regularly interspaced short palindromic repeats; IHC, immunohistochemistry; LAG-3, lymphocyte-activation gene 3; NGS, next-generation sequencing; WT, wild type.

20

Supplemental material

BMJ Publishing Group Limited (BMJ) disclaims all liability and responsibility arising from any reliance placed on this supplemental material which has been supplied by the author(s)

J Clin Pathol

REFERENCE

1.   Jung K, Lee J, Gupta V, et al. Comparison of bootstrap confidence interval methods for GSCA using a Monte Carlo simulation. *Front Psychol* 2019;10:2215.