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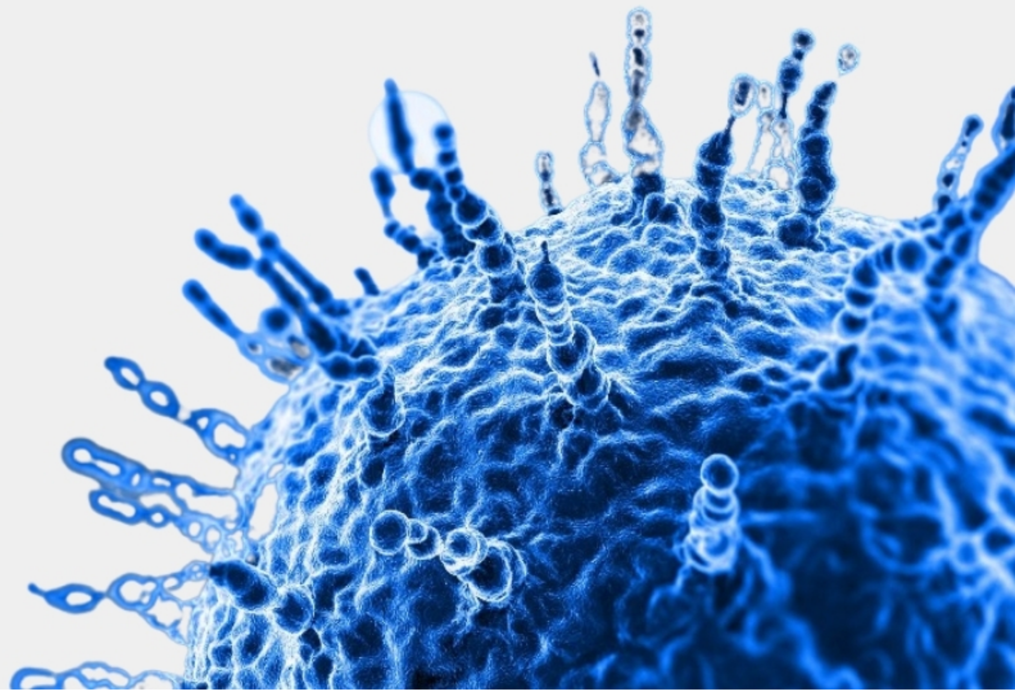

Histopathology

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False-negative programmed death-ligand 1 immunostaining in ethanol-fixed endobronchial ultrasound-guided transbronchial needle aspiration specimens of non-small-cell lung cancer patients

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False-negative programmed death-ligand 1 immunostaining in ethanol-fixed endobronchial ultrasound-guided transbronchial needle aspiration specimens of non-small-cell lung cancer patients

Aims: Programmed death-ligand 1 (PD-L1) immunostaining is used to predict which non-small-cell lung cancer (NSCLC) patients will respond best to treatment with programmed cell death protein 1/PD-L1 inhibitors. PD-L1 immunostaining is sometimes performed on alcohol-fixed cytological specimens instead of on formalin-fixed paraffin-embedded (FFPE) biopsies or resections. We studied whether ethanol prefixation of clots from endobronchial ultrasound-guided transbronchial needle aspiration (EBUS-TBNA) results in diminished PD-L1 immunostaining as compared with formalin fixation.

Methods and results: FFPE cell blocks from EBUS-TBNA specimens of 54 NSCLC patients were identified. For each case, paired samples were available, consisting of clots directly immersed in formalin and clots prefixed in Fixcyt (50% ethanol). Serial sections were immunostained for PD-L1 by use of the standardised SP263 assay and the 22C3 antibody as a laboratory-developed test (LDT). PD-L1 positivity was determined with two cut-offs (1% and 50%). Concordance of PD-L1 positivity between the formalin-fixed (gold standard) and ethanol-prefixed material was assessed. When the 22C3 LDT was used, 30% and 36% of the ethanol-prefixed specimens showed false-negative results at the 1% and 50% cut-offs, respectively (kappa 0.64 and 0.68). When SP263 was used, 22% of the ethanol-prefixed specimens showed false-negative results at the 1% cut-off (kappa 0.67). At the 50% cut-off, concordance was higher (kappa 0.91), with 12% of the ethanol-prefixed specimens showing false-negative results.

Conclusion: Ethanol fixation of EBUS-TBNA specimens prior to formalin fixation can result in a considerable number of false-negative PD-L1 immunostaining results when a 1% cut-off is used and immunostaining is performed with SP263 or the 22C3 LDT. The same applies to use of the 50% cut-off when immunostaining is performed with the 22C3 LDT.

Keywords: 22C3 antibody, cytology, immunohistochemistry, immunotherapy, non-small-cell lung carcinoma, programmed cell death-ligand 1, SP263 antibody, tissue fixation

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Introduction

Immunotherapy with checkpoint blockade has become an integral part of cancer treatment, with several immune checkpoint inhibitors having been registered for various forms of cancer.[1] In patients with advanced non-small-cell lung cancer (NSCLC), inhibition of programmed cell death protein 1 (PD-1) or its ligand programmed death-ligand 1 (PD-L1) may lead to clinical benefit.[2–7] Registered drugs are the PD-1 inhibitors nivolumab and pembrolizumab and the PD-L1 inhibitors atezolizumab and durvalumab. Various clinical trials have shown better clinical results or a trend for better efficacy of PD-1 and PD-L1 inhibitors in patients with higher expression of PD-L1 on tumour cells, as measured with immunohistochemistry (IHC).[2,3,5,6,8,9] In clinical practice, therefore, pathologists determine the percentage of tumour cells that show PD-L1 expression, also known as the tumour proportion score (TPS), which is used to predict which patients might respond best to treatment with PD-1 or PD-L1 inhibitors.

In predicting these chances of response to anti-PD-1 and anti-PD-L1 therapeutics, two cut-offs for the PD-L1 TPS are clinically relevant. A cut-off of 50% is relevant for prescription of pembrolizumab. This drug may be prescribed as first-line therapy combined with chemotherapy to patients with stage IV NSCLC (without *EGFR* or *ALK* mutations), regardless of PD-L1 expression.[9,10] However, patients whose tumours show a TPS of ≥50% may receive first-line pembrolizumab monotherapy,[9,10] exposing them to far less treatment toxicity. Second, the 1% cut-off is used for prescription of durvalumab in various European countries, as the European Medicines Agency has advised prescription of this drug as consolidation treatment to stage III NSCLC patients whose tumours show a TPS of ≥1%.[11] The PD-1/PD-L1 inhibitors nivolumab and atezolizumab may be prescribed as second-line treatment to patients with metastatic NSCLC regardless of PD-L1 expression.[9] However, measurement of PD-L1 expression could aid in predicting the chances of response to these drugs as well, and may in that way guide clinicians in their treatment decisions for the individual patient.[12]

The efficacy of PD-1/PD-L1 inhibitors in relation to PD-L1 expression was determined in clinical trials that performed IHC on formalin-fixed paraffin-embedded (FFPE) surgical biopsies or resections.[13] However, because a large proportion of NSCLC patients are diagnosed at an advanced stage of disease, diagnosis and treatment decisions are often based on cytological specimens from readily accessible sites.[14] Because of their minimally invasive character, techniques such as endobronchial ultrasound-guided transbronchial needle aspiration (EBUS-TBNA) and transthoracic fine needle aspiration are preferable in this setting.[14,15] The material collected during these procedures can be fixed and processed into cell blocks in a great variety of ways, which may quite often not result in FFPE material.[16] As the use of PD-L1 antibodies for immunostaining on cytological specimens fixed and processed in various ways has not been validated in clinical trials, it is unclear whether the results of PD-L1 immunostaining on these specimens can adequately predict response to PD-1/PD-L1 inhibitors.

So far, there have been several studies assessing the concordance of PD-L1 immunostaining between surgical biopsies or resections and cytological cell blocks. Most of these have concluded that PD-L1 testing can safely be performed on the latter, on the basis of high levels of concordance between histology and cell blocks.[13,17–20] However, these studies used formalin-fixed cytological material, whereas, in clinical practice, laboratories may use alcohol (methanol or ethanol)-based fixatives or transport media.[21] A potential advantage of using alcohols such as ethanol for fixation is their beneficial effect on nucleic acid preservation as compared with formalin fixation, resulting in higher-quality material for molecular analysis.[22] However, it is known from other studies that alcohol fixation can result in decreased intensity of immunostaining for various antibodies.[23–27] Potentially, alcohol fixation could have a similar negative effect on PD-L1 immunostaining. This could have an important impact on the selection of NSCLC patients who are eligible for immunotherapy, potentially leading to denial of treatment options that these patients could benefit from. In this study, the effect of ethanol prefixation followed by delayed formalin fixation on PD-L1 immunostaining was determined, by the use of paired formalin-fixed and ethanol-fixed EBUS-TBNA samples from NSCLC patients.

Materials and methods

PATIENTS AND SPECIMENS

For this study, NSCLC patients who had undergone EBUS-TBNA on lymph nodes or primary tumours at the Canisius-Wilhelmina Hospital (CWZ) (Nijmegen, The Netherlands) between November 2015 and August 2018 were identified retrospectively. Aspirated material from all patients was split into two separate fixatives, with approximately two-thirds of

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the material (the yield from sampling the lymph node or primary tumour twice) being collected in 20 ml of 10% neutral buffered formalin, and one-third of the material (the yield from sampling once) being collected in 20 ml of Fixcyt (50% ethanol and 2% polyethylene glycol solution). The average prefixation duration was 1 h, with a maximum of 2 h. Upon arrival of the EBUS-TBNA material at the pathology laboratory, visible clots from the material fixed in Fixcyt were directly placed into formalin for further fixation, with postfixation times ranging from 3 h to 24 h. The total fixation duration (formalin fixation only or Fixcyt fixation followed by formalin fixation) was equal between the formalin material and Fixcyt material collected from the same patient. Clots from both the formalin-fixed and Fixcyt-fixed aspirated material were then processed into paraffin-embedded blocks, with the same routine biopsy processing programme being used for both specimens. Both the formalin-fixed and Fixcyt-fixed blocks from each patient were collected from the pathology archive. The study was approved by the institutional review board at CWZ. Data and tissue samples were all handled according to the General Data Protection Regulation. All patient material was used anonymously, and collection and use of material was in accordance with the code of conduct for the responsible use of residual human tissue for research, established by the Federation of Dutch Medical Scientific Societies.[28]

PD-L1 IMMUNOSTAINING

Consecutive 3-μm sections were cut from both formalin-fixed and Fixcyt-fixed blocks of all included patients. Sections were stained with haematoxylin and eosin (H&E) and with two PD-L1 antibodies, i.e. Ventana (Ventana Medical Systems, Inc., Tucson, Arizona, U.S.A.) SP263 and Dako (Agilent Technologies, Santa Clara, California, U.S.A.) 22C3. The H&E-stained sections were used to determine the number of tumour cells present in each FFPE block, with exclusion of all patients with one or more blocks containing <100 viable tumour cells. For immunostaining with SP263, a standardised assay was used on the Ventana Benchmark Ultra platform at Utrecht University Medical Centre (UMCU) (Utrecht, The Netherlands), according to the manufacturer's instructions. On the basis of retrospective analysis of pathology reports generated in 2017 and 2018, the average PD-L1 positivity rates in histological material of NSCLC patients at UMCU were 57% and 28% at the 1% cut-off and at the 50% cut-off, respectively. Similar PD-L1 expression rates have been reported in a large series of NSCLC specimens.[29] The 22C3 antibody was used at CWZ in a laboratory-developed test (LDT) on the Dako Omnis platform [dilution 1:25, 30 min of incubation; heat-induced epitope retrieval (97°C) with low-pH buffer; EnVision FLEX+ (Agilent Technologies, Santa Clara, California, U.S.A.) mouse LINKER detection kit; EnVision FLEX DAB Enhancer (5 min)], validated for use in clinical practice through comparison of sections from 100 NSCLC patients with sections from the same patients immunostained for PD-L1 in an academic referral hospital (all FFPE tissue sections). The 22C3 LDT has shown good to excellent results in several external quality assessment schemes performed by NordiQC and the European Society of Pathology. The average PD-L1 positivity rates were 69% at the 1% cut-off and 39% at the 50% cut-off, on the basis of data from 2017 and 2018. The rate at the 50% cut-off is higher than the positivity rates reported in the Keynote trials that assessed the efficacy of pembrolizumab,[4,5] but positivity rates up to 42.7% have been reported by others.[30]

ASSESSMENT OF PD-L1 IMMUNOSTAINING

All PD-L1-immunostained slides containing ≥100 viable tumour cells were scored by an experienced pathologist, certified for PD-L1 scoring, together with a pathology resident, under a double-headed microscope. The TPS was established by determining the percentage of PD-L1-positive tumour cells relative to the total number of tumour cells. Membranous immunostaining of any intensity was considered to be valid, whereas cytoplasmic immunostaining and immunostaining of necrotic tumour cells were disregarded. The TPS was determined with scores of 0%, 1%, 5% or 10% for the TPS ranging from 0% to 10%. For scores of >10%, a 10% increment was used. Scoring of all slides immunostained with one antibody was performed within one session. Scoring of all slides immunostained with the other antibody was performed in a second session, with a period of 3 weeks between the scoring sessions and rearrangement of the order of slides before the second session. The scorers were blinded to the fixative that was used, and did not know which slides belonged to the same patient.

STATISTICAL ANALYSIS

The concordance of PD-L1 immunostaining between formalin-fixed and Fixcyt-fixed material was assessed by calculating intraclass correlation coefficients (ICCs) for continuous PD-L1 scores. These scores were then dichotomised according to two clinically relevant cut-

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offs for the TPS, i.e. ≥1% and ≥50%. The overall percentage agreement (OPA), the positive percentage agreement (PPA) and the negative percentage agreement were calculated for both cut-offs, with the material fixed in formalin being used as a reference standard. On the basis of the guideline on principles of analytical validation of immunohistochemical assays from the College of American Pathologists (CAP) Pathology and Laboratory Quality Center, an overall agreement of at least 90% between the formalin-fixed and Fixcyt-fixed material was regarded as acceptable.[31] Cohen's kappa (κ) values were also determined. According to McHugh's suggested interpretation of the kappa statistic for clinical laboratories, values ≥0.80 were considered to indicate strong agreement and were deemed to be acceptable for clinical practice.[32] Statistical analysis was performed with RSTUDIO version 1.1.456 and IBM SPSS STATISTICS version 25.

Results

PATIENT AND SPECIMEN CHARACTERISTICS

Formalin-fixed and Fixcyt-fixed blocks from 67 NSCLC patients were collected. Of these, 54 cases were eligible for inclusion. The remaining 13 cases had insufficient numbers of viable tumour cells (<100) in one or both blocks, and were therefore excluded. Patient and specimen characteristics of the included cases are shown in Table 1.

When a cut-off of ≥1% was used to determine PD-L1 positivity, 33 (61%) and 32 (59%) formalin-fixed specimens, respectively, were PD-L1-positive when immunostaining was performed with the 22C3 LDT and with SP263. With the same cut-off, 23 (43%) and 27 (50%) Fixcyt-fixed specimens were PD-L1-positive. With a cut-off of ≥50%, 14 (26%) formalin-fixed specimens were PD-L1-positive when immunostaining was performed with the 22C3 LDT, and 16 (30%) were PD-L1-positive when immunostaining was performed with SP263. Of the Fixcyt-fixed specimens, 10 (19%) and 14 (26%), respectively, showed a TPS of ≥50% when immunostaining was performed with the 22C3 LDT and with SP263.

COMPARISON OF PD-L1 IMMUNOSTAINING BETWEEN FORMALIN-FIXED AND FIXCYT-FIXED SPECIMENS WHEN IMMUNOSTAINING WAS PERFORMED WITH THE 22C3 LDT

When the concordance of the TPS was assessed on a continuous scale for material immunostained by use of the 22C3 LDT, the correlation between the formalin-fixed and Fixcyt-fixed specimens was on the boundary between moderate and good [ICC 0.76; 95% confidence interval (CI) 0.60–0.86].[33] However, dichotomisation of the TPS at the 1% cut-off and the 50% cut-off resulted in lower concordance levels (Cohen's κ of 0.64 and 0.68, respectively, and OPAs of <90%) (Table 2). Differences in categorisation of the TPS between the two types of material, with both cut-offs, are shown in Figure 1A. When the 1% cut-off was used to determine PD-L1 positivity, 10 (30%) of 33 cases that were PD-L1-positive in the formalin-fixed specimen showed false-negative results in the Fixcyt-fixed specimen, resulting in a PPA of only 70%. Figure 2A,B shows a representative example of a case with a TPS of ≥1% in the formalin-fixed

Table 1. Patient and specimen characteristics

Characteristic	*n* (%)
Diagnosis	
Adenocarcinoma	32 (59)
Squamous cell carcinoma	15 (28)
NSCLC NOS	6 (11)
Adenosquamous carcinoma	1 (2)
TBNA source	
Lymph node	47 (87)
Primary tumour	7 (13)
PD-L1 expression (TPS) formalin ≥1%	
22C3 LDT	33 (61)
SP263	32 (59)
PD-L1 expression (TPS) formalin ≥50%	
22C3 LDT	14 (26)
SP263	16 (30)
PD-L1 expression (TPS) Fixcyt ≥1%	
22C3 LDT	23 (43)
SP263	27 (50)
PD-L1 expression (TPS) Fixcyt ≥50%	
22C3 LDT	10 (19)
SP263	14 (26)

LDT, laboratory-developed test; NOS, not otherwise specified; NSCLC, non-small-cell lung cancer; PD-L1, programmed death-ligand 1; TBNA, transbronchial needle aspiration; TPS, tumour proportion score.

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Table 2. Concordance of programmed death-ligand 1 (PD-L1) positivity between specimens fixed in formalin and specimens fixed in Fixcyt for SP263 and the 22C3 laboratory-developed test (LDT), with two different cut-offs to determine PD-L1 positivity (≥1% and ≥50%)

	Concordance when a 1% cut-off was used				Concordance when a 50% cut-off was used			
	OPA (%)	PPA (%)	NPA (%)	Cohen's kappa (95% CI)	OPA (%)	PPA (%)	NPA (%)	Cohen's kappa (95% CI)
22C3 LDT	81	70	100	0.64 (0.45–0.83)	89	64	98	0.68 (0.44–0.92)
SP263	83	78	91	0.67 (0.47–0.87)	96	88	100	0.91 (0.78–1.00)

CI, confidence interval; NPA, negative percentage agreement; OPA, overall percentage agreement; PPA, positive percentage agreement.

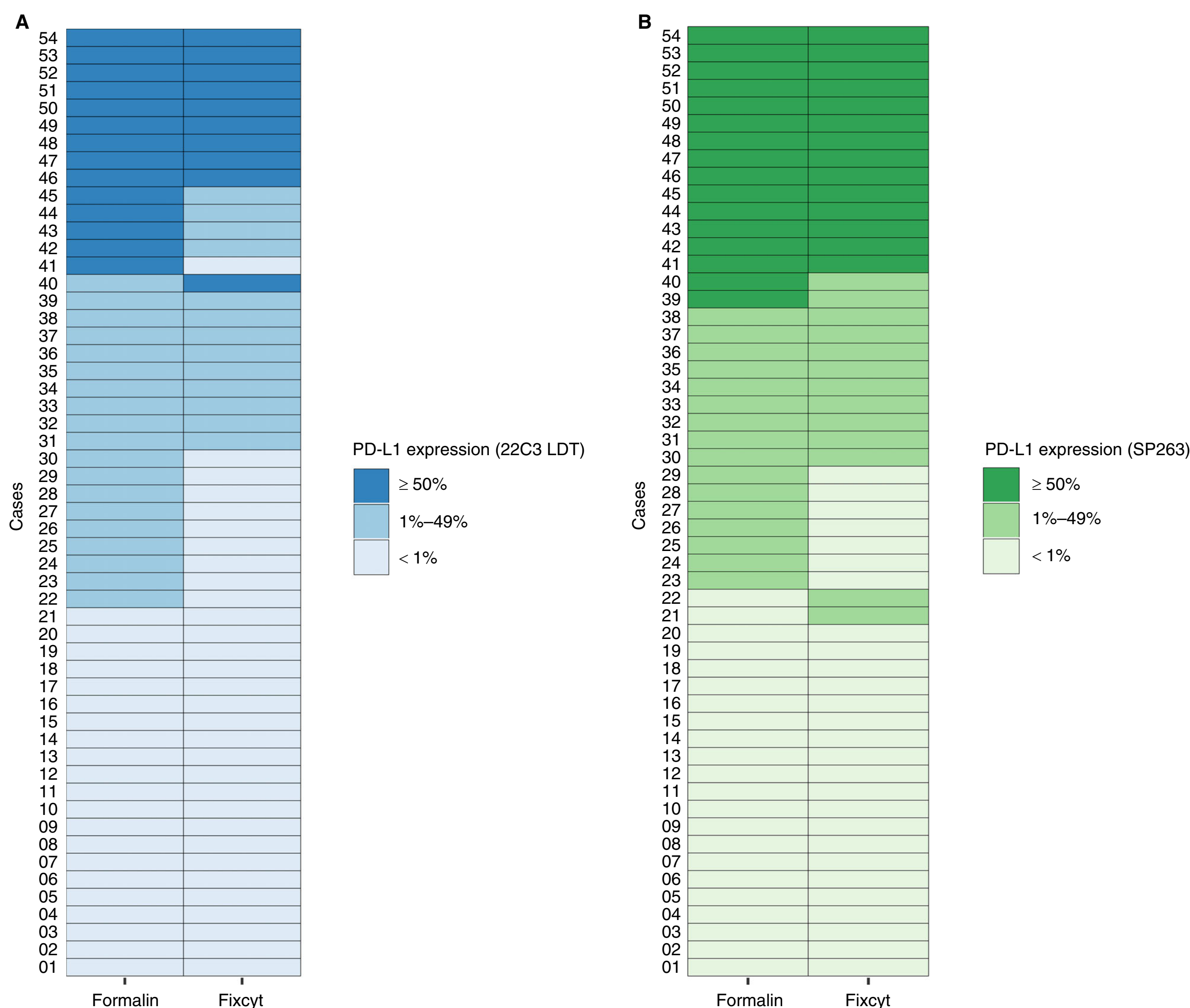

Figure 1. The programmed death-ligand 1 (PD-L1) tumour proportion score (TPS) in three categories for formalin-fixed and Fixcyt-fixed material per case, determined with the 22C3 laboratory-developed test (LDT) (**A**) and the SP263 standardised assay (**B**). Cases for which colours do not correspond showed discordant TPS values between formalin-fixed and Fixcyt-fixed material.

specimen and a TPS of <1% in the Fixcyt-fixed specimen. When the 50% cut-off was used, the PPA was only 64%, owing to five (36%) of 14 cases showing false-negative results in the Fixcyt-fixed specimens. Figure 3A,B shows a representative example of a case with a TPS of ≥50% in the formalin-fixed specimen

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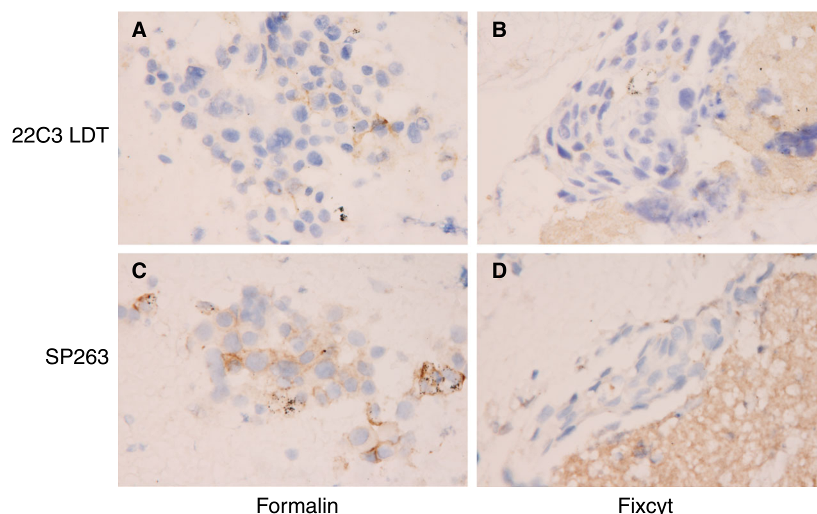

Figure 2. Images of the programmed death-ligand 1 (PD-L1) immunostaining pattern of an exemplary case showing a tumour proportion score (TPS) of ≥1% in formalin-fixed material when immunostaining was performed with the 22C3 laboratory-developed test (LDT) (**A**) and with SP263 (**C**). The Fixcyt-fixed material showed a TPS of <1% when immunostaining was performed with the 22C3 LDT (**B**) and with SP263 (**D**).

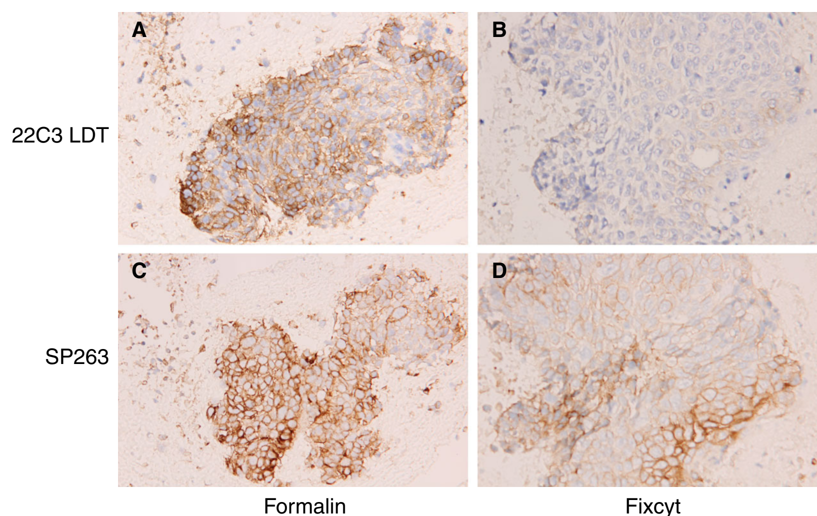

Figure 3. Images of the programmed death-ligand 1 (PD-L1) immunostaining pattern of an exemplary case showing a tumour proportion score (TPS) of ≥50% in formalin-fixed material when immunostaining was performed with the 22C3 laboratory-developed test (LDT) (**A**) and with SP263 (**C**). The Fixcyt-fixed material showed a TPS of <50% when immunostaining was performed with the 22C3 LDT (**B**) and a TPS of ≥50% when immunostaining was performed with SP263 (**D**).

and a TPS of <50% in the Fixcyt-fixed specimen when immunostaining for PD-L1 was performed with the 22C3 LDT.

COMPARISON OF PD-L1 IMMUNOSTAINING BETWEEN FORMALIN-FIXED AND FIXCYT-FIXED SPECIMENS WHEN IMMUNOSTAINING WAS PERFORMED WITH SP263

When immunostaining was performed with SP263, analysis of the concordance of the TPS on a continuous scale showed high agreement (ICC 0.92; 95% CI 0.90–0.95). Again, however, dichotomisation of the TPS at the 1% cut-off resulted in much lower concordance levels (OPA of <90% and Cohen's κ of 0.67) (Table 2). Figure 1B shows that, when this cut-off was used, seven (22%) of 32 cases that were PD-L1-positive in the formalin-fixed specimen showed false-negative results in the Fixcyt-fixed specimen (PPA of 78%). A representative example of this phenomenon (lower intensity of PD-L1 immunostaining in Fixcyt-fixed material than in formalin-fixed

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material when SP263 was used) is shown in Figure 2C,D. Dichotomisation of the TPS at the 50% cut-off resulted in much higher concordance levels than those found when the 1% cut-off was used (Cohen's κ of 0.91 versus 0.67 and PPA of 88% versus 78%) (Table 2). Figure 3C,D shows a representative example of a case in which the PD-L1 immunostaining was scored as ≥50% in both the formalin-fixed and the Fixcyt-fixed material when SP263 was used.

COMPARISON OF PD-L1 IMMUNOSTAINING BETWEEN THE 22C3 LDT AND SP263

See Doc. S1 for the results of a comparison of PD-L1 immunostaining between the 22C3 LDT and SP263 for both formalin-fixed and Fixcyt-fixed material.

Discussion

In this retrospective study, the effect of prefixation in an ethanol-based fixative on PD-L1 immunostaining was studied with two PD-L1 immunohistochemical assays (the 22C3 LDT and the SP263 standardised assay) validated for use on FFPE tissue. It was shown that fixation of EBUS-TBNA material in Fixcyt (ethanol-based) prior to formalin fixation resulted in a considerable number of false-negative PD-L1 immunostaining results when the 1% cut-off was used to determine PD-L1 positivity. When PD-L1 positivity was determined with the 50% cut-off, immunostaining by use of the 22C3 LDT again resulted in a substantial number of false-negative PD-L1 immunostaining results in the Fixcyt-fixed material, whereas this was not the case for SP263.

Few other studies have assessed the influence of ethanol (pre)fixation on PD-L1 immunostaining results in cytology cell blocks. Wang *et al.*[34] concluded that alcohol fixation (methanol or ethanol) does not affect PD-L1 immunostaining. However, no comparisons between paired samples of the same patients were made, and the authors state that it is possible that most of the cases fixed in both alcohol and formalin had short alcohol prefixation times. A study by Gosney *et al.*,[35] which did use paired samples of aspirates fixed in alcohol-based fixatives and formalin, also reported no effect of the use of alcohol-based fixatives on the expression of PD-L1 or its interpretation. Most of the alcohol-fixed samples in the study, however, were fixed in CytoRich Red, a solution that also contains formaldehyde. These samples were therefore exposed to more formalin during the processing procedure than if they had been fixed in an ethanol-based fixative such as Fixcyt, which could potentially explain the differences in results from those of our own study. A study by Jain *et al.*[36] found an overall concordance of PD-L1 immunostaining of 88.4% between small biopsies and matched liquid-based cytology smears, but, similarly to the procedure of Gosney *et al.*,[35] these smears were fixed in CytoRich Red. Similarly to our findings, Lloyd *et al.* showed a negative effect of CytoLyt (a methanol-based fixative) on PD-L1 immunostaining in PD-L1-expressing cell lines.[37]

The occurrence of false-negative PD-L1 immunostaining results is problematic, as it could lead to denial of potentially beneficial treatment options to patients with NSCLC. The use of EBUS-TBNA specimens for PD-L1 testing is most often seen in patients who present with locally advanced or metastatic disease at diagnosis. These patients are precisely the ones who could benefit from treatment with PD-1/PD-L1 inhibitors. It is thus of the utmost importance that pathologists can accurately determine the PD-L1 TPS for these patients. Hence, pathologists should be aware of the risks of using an ethanol-based (pre)fixative. The concordance levels of the PD-L1 TPS were lowest for both the 22C3 LDT and SP263 with use of the 1% cut-off, indicating that the use of ethanol-based fixatives could result in the wrongful denial of durvalumab as consolidation treatment to stage III NSCLC patients in clinical practice in various European countries.[11] The combination of Fixcyt-fixed material and immunostaining by use of the 22C3 LDT also resulted in disappointing concordance levels when a 50% cut-off was used. This could result in stage IV NSCLC patients being prescribed a more toxic first-line treatment regimen of pembrolizumab combined with chemotherapy, whereas they could have been treated with a less toxic treatment option consisting of pembrolizumab alone.[9,10]

Interestingly, the use of SP263 resulted in high concordance of the PD-L1 TPS between formalin-fixed and Fixcyt-fixed material when the 50% cut-off was used for dichotomisation. Overall, slightly stronger immunostaining intensity was seen in sections immunostained with SP263 than in those immunostained by use of the 22C3 LDT, with discordant cases more often showing a lower TPS when the 22C3 LDT was used than when SP263 was used than the other way around. A decrease in immunostaining intensity due to ethanol (pre)fixation might therefore be less problematic when SP263 is used than when the 22C3 LDT is used. It could also be that the negative effect of ethanol (pre)fixation on PD-L1

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immunoreactivity is smaller with the use of SP263 than with use of the 22C3 LDT. Although these two antibodies target the same protein, they do target different epitopes of this protein.[38] Perhaps the alteration in tertiary structure of the PD-L1 protein caused by alcohol fixation[21] results in one epitope being more capable of binding with the PD-L1 antibody than the other. A study by Munari *et al.*[39] also showed a high concordance rate between ethanol-fixed material (cytological smears) and FFPE surgical resection material at the 50% cut-off, when the SP263 standardised assay was used. Scoring of PD-L1 positivity with the 50% cut-off thus seems to be feasible on ethanol-prefixed material when SP263 is used. However, we would suggest assessing the concordance between formalin-fixed and ethanol-prefixed material in more 'critical samples', i.e. samples that show a PD-L1 TPS closer to the threshold of PD-L1 positivity,[40] before drawing a more definitive conclusion on the actual feasibility of using SP263 on ethanol-prefixed material to determine PD-L1 positivity at a 50% cut-off in clinical practice.

It has been described previously that alcohol fixation leads to false-negative immunostaining results when conventional IHC protocols, validated for FFPE tissue, are used.[23–27,41] Nevertheless, in clinical practice, cell blocks are often seen as ideal for immunostaining,[42] even though different fixatives, including alcohol-based fixatives, may be used in preparing them. Fowler and Lachar state that it is a common mistake in cytology laboratories to not carefully examine the cell block methodology and its potential negative impact on IHC interpretation.[43] They advise comparison of immunostaining results between cytological samples and surgical pathology samples, prior to the introduction of any new cell block method.[43] This is in line with the guideline from the CAP, which states that laboratories should test a sufficient number of cases to ensure that IHC assays achieve the expected results, if they are used on cytological specimens that are not processed in the same manner as the tissues used for assay validation.[31] Similarly, Rekhtman *et al.*,[44] who recently developed a modified HistoGel-based cell block preparation method that includes the addition of 95% ethanol, state that laboratories who adopt their method should consider revalidation of immunostains. In the literature, there are some examples demonstrating that changing an IHC protocol can lead to good immunostaining results when alcohol-fixed specimens are used, whereas the standard IHC protocol, validated for FFPE tissue, showed reduced immunostaining intensity.[23] However, in clinical practice, many laboratories use standardised PD-L1 assays, such as the standardised SP263 assay and the 22C3 pharmDx assay, which received Food and Drug Administration approval and/or CE-IVD marking for use only with standardised protocols designed by the manufacturers.[45–47] Moreover, changing an IHC protocol does not always result in improved immunostaining,[23,25] leaving it unsure whether changing PD-L1 IHC protocols would actually result in fewer false-negative immunostaining results when they are used on ethanol-(pre)fixed specimens.

This study has some limitations. First, the sample size is rather small. Because of the retrospective nature of this study, the availability of patient material was dependent on the number of NSCLC patients who had actually undergone EBUS-TBNA in clinical practice and had their aspirated material fixed in both Fixcyt and formalin. However, we do believe that the design of this study, which used paired samples to compare PD-L1 immunostaining between ethanol-prefixed and formalin-fixed material, provides a valuable contribution to the current literature. Second, we used the cell blocks fixed in formalin as a reference standard in our analyses. Ideally, we would have liked to use true histological specimens as the gold standard for our comparisons. Unfortunately, matched histological specimens were not available for our study cases. Third, we only used two PD-L1 antibodies in our study, whereas, in clinical practice, laboratories may use other PD-L1 antibodies, such as 28-8 in the pharmDx assay or E1L3N. We cannot draw any conclusions regarding the effect of ethanol-based fixation on PD-L1 immunostaining with antibodies and protocols other than the ones that we used. Fourth, the samples in our study were fixed in ethanol for only a short period of time, with a maximum of 2 h. In clinical practice, aspirated material might sometimes rest in a fixative for a longer amount of time, especially when it is collected just before or during the weekend. Potentially, a longer duration of ethanol (pre)fixation could result in even more detrimental effects on PD-L1 immunostaining. Also, we cannot draw definitive conclusions on the effect of formalin postfixation after alcohol fixation on PD-L1 immunostaining, as we did not know the individual fixation times for the samples in our study, and were therefore unable to determine whether there was any difference in PD-L1 results between specimens with shorter and longer formalin postfixation times. Fifth, the uneven distribution of aspirated material between formalin and Fixcyt could have played a role in causing discrepancies between formalin-fixed and Fixcyt-fixed samples in our study, as the Fixcyt-fixed material might have contained a smaller number of tumour cells. We did not,

however, observe prominent differences in the numbers of tumour cells between the two types of specimen when scoring PD-L1. Moreover, nearly one-third of the excluded cases were excluded because the formalin-fixed cell blocks did not contain enough tumour cells, whereas the Fixcyt-fixed cell blocks did. We therefore do not believe that the number of tumour cells was systematically lower in the Fixcyt-fixed specimens, diminishing the influence that the uneven distribution of the aspirated material would have had on creating discordance in PD-L1 immunostaining between the different specimen types. Finally, both intratumoral heterogeneity and intraobserver variability could potentially explain part of the discordance seen between formalin-fixed and Fixcyt-fixed material, which is also indicated by the three cases that showed higher PD-L1 expression in the Fixcyt-fixed specimen than in the formalin-fixed specimen (two cases when immunostaining was performed with SP263; one case when immunostaining was performed with the 22C3 LTD). However, as the overall discordance that we observed mainly consisted of false-negative results in Fixcyt-fixed material as compared with formalin-fixed material, rather than a mix of both false-negative and false-positive results, it seems highly unlikely that the discordance could be explained solely by the presence of intratumoral heterogeneity or intraobserver variability.

To conclude, when SP263 and 22C3 IHC protocols, validated for use on FFPE material, are used on cytological specimens prefixed in an ethanol-based fixative, this results in a considerable number of false-negative PD-L1 immunostaining results. This occurs when both the 1% and the 50% cut-offs are used to determine PD-L1 positivity, although the risk of false-negative results seems to be smallest when the SP263 standardised assay is used and scoring of PD-L1 positivity is performed with the 50% cut-off. Pathologists should be aware that scoring of PD-L1 expression as negative on the basis of standard PD-L1 IHC protocols used on specimens (pre)fixed in ethanol could lead to patients wrongfully being denied treatment options that they could actually benefit from.

Conflicts of interest

B. M. Koomen and S. M. Willems report research grants from AstraZeneca, MSD, and Roche Diagnostics, outside the submitted work. S. M. Willems also reports research grants from Amgen, Bayer, BMS, NextCure, and Pfizer, outside the submitted work. The other authors declare that they have no conflicts of interest.

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Author contributions

B. M. Koomen and W. Vreuls designed the study, performed the research, and analysed the data. B. M. Koomen wrote the paper, with major contributions from W. Vreuls. M. de Boer helped to design the study and analyse the data, and provided feedback on the manuscript. E. J. de Ruiter helped to analyse the data and provided feedback on the manuscript. J. Hoelters collected data and provided feedback on the manuscript. A. Vink helped to design the study and analyse the data, and provided feedback on the manuscript. S. M. Willems helped to design the study and analyse the data, and provided feedback on the manuscript.

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References

1. Ribas A, Wolchok JD. Cancer immunotherapy using checkpoint blockade. *Science* 2018; **359**; 1350–1355.
2. Borghaei H, Paz-Ares L, Horn L *et al*. Nivolumab versus docetaxel in advanced nonsquamous non-small-cell lung cancer. *N. Engl. J. Med.* 2015; **373**; 1627–1639.
3. Brahmer J, Reckamp KL, Baas P *et al*. Nivolumab versus docetaxel in advanced squamous-cell non-small-cell lung cancer. *N. Engl. J. Med.* 2015; **373**; 123–135.
4. Reck M, Rodríguez-Abreu D, Robinson AG *et al*. Pembrolizumab versus chemotherapy for PD-L1-positive non-small-cell lung cancer. *N. Engl. J. Med.* 2016; **375**; 1823–1833.
5. Herbst RS, Baas P, Kim D-W *et al*. Pembrolizumab versus docetaxel for previously treated, PD-L1-positive, advanced non-small-cell lung cancer (KEYNOTE-010): a randomised controlled trial. *Lancet* 2016; **387**; 1540–1550.
6. Rittmeyer A, Barlesi F, Waterkamp D *et al*. Atezolizumab versus docetaxel in patients with previously treated non-small-cell lung cancer (OAK): a phase 3, open-label, multicentre randomised controlled trial. *Lancet* 2017; **389**; 255–265.
7. Antonia SJ, Villegas A, Daniel D *et al*. Durvalumab after chemoradiotherapy in stage III non-small-cell lung cancer. *N. Engl. J. Med.* 2017; **377**; 1919–1929.

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8. Mok TSK, Wu YL, Kudaba I *et al*. Pembrolizumab versus chemotherapy for previously untreated, PD-L1-expressing, locally advanced or metastatic non-small-cell lung cancer (KEYNOTE-042): a randomised, open-label, controlled, phase 3 trial. *Lancet* 2019; **393**; 1819–1830.
9. Planchard D, Popat S, Kerr K *et al*. Metastatic non-small cell lung cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. *Ann. Oncol.* 2018; **29**(Suppl. 4); iv192–iv237.
10. European Society for Medical Oncology (ESMO). Metastatic non-small-cell lung cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. 2019. Available at: https://www.esmo.org/Guidelines/Lung-and-Chest-Tumours/Metastatic-Non-Small-Cell-Lung-Cancer (accessed 11 November 2019).
11. EMA. Imfinzi (Durvalumab). 2018. Available at: https://www.ema.europa.eu/en/medicines/human/EPAR/imfinzi (accessed 25 September 2019).
12. Scheerens H, Malong A, Bassett K *et al*. Current status of companion and complementary diagnostics: strategic considerations for development and launch. *Clin. Transl. Sci.* 2017; **10**; 84–92.
13. Russell-Goldman E, Kravets S, Dahlberg SE, Sholl LM, Vivero M. Cytologic-histologic correlation of programmed death-ligand 1 immunohistochemistry in lung carcinomas. *Cancer Cytopathol.* 2018; **126**; 253–263.
14. Skov BG, Høgdall E, Clementsen P *et al*. The prevalence of EGFR mutations in non-small cell lung cancer in an unselected Caucasian population. *APMIS* 2015; **123**; 108–115.
15. Roh MH. The utilization of cytologic fine-needle aspirates of lung cancer for molecular diagnostic testing. *J. Pathol. Transl. Med.* 2015; **49**; 300–309.
16. Nambirajan A, Jain D. Cell blocks in cytopathology: an update. *Cytopathology* 2018; **29**; 505–524.
17. Skov BG, Skov T. Paired comparison of PD-L1 expression on cytologic and histologic specimens from malignancies in the lung assessed with PD-L1 IHC 28–8pharmDx and PD-L1 IHC 22C3pharmDx. *Appl. Immunohistochem. Mol. Morphol.* 2017; **25**; 453–459.
18. Ilie M, Juco J, Huang L, Hofman V, Khambata-Ford S, Hofman P. Use of the 22C3 anti-programmed death-ligand 1 antibody to determine programmed death-ligand 1 expression in cytology samples obtained from non-small cell lung cancer patients. *Cancer Cytopathol.* 2018; **126**; 264–274.
19. Sakakibara R, Inamura K, Tambo Y *et al*. EBUS-TBNA as a promising method for the evaluation of tumor PD-L1 expression in lung cancer. *Clin. Lung Cancer* 2017; **18**; 527–534.
20. Hernandez A, Brandler TC, Zhou F, Moreira AL, Schatz-Siemers N, Simsir A. Assessment of programmed death-ligand 1 (PD-L1) immunohistochemical expression on cytology specimens in non-small cell lung carcinoma. *Am. J. Clin. Pathol.* 2019; **151**; 403–415.
21. Eltoum I, Fredenburgh J, Myers RB, Grizzle WE. Introduction to the theory and practice of fixation of tissues. *J. Histotechnol.* 2001; **24**; 173–190.
22. Chung J-Y, Song JS, Ylaya K *et al*. Histomorphological and molecular assessments of the fixation times comparing formalin and ethanol-based fixatives. *J. Histochem. Cytochem.* 2018; **66**; 121–135.
23. Sauter JL, Grogg KL, Vrana JA, Law ME, Halvorson JL, Henry MR. Young investigator challenge: validation and optimization of immunohistochemistry protocols for use on cellient cell block specimens. *Cancer Cytopathol.* 2016; **124**; 89–100.
24. Gruchy JR, Barnes PJ, Dakin Hache KA. CytoLyt(R) fixation and decalcification pretreatments alter antigenicity in normal tissues compared with standard formalin fixation. *Appl. Immunohistochem. Mol. Morphol.* 2015; **23**; 297–302.
25. Buonocore DJ, Konno F, Jungbluth AA *et al*. CytoLyt fixation significantly inhibits MIB1 immunoreactivity whereas alternative Ki-67 clone 30–9 is not susceptible to the inhibition: critical diagnostic implications. *Cancer Cytopathol.* 2019; **127**; 643–649.
26. Gorman BK, Kosarac O, Chakraborty S, Schwartz MR, Mody DR. Comparison of breast carcinoma prognostic/predictive biomarkers on cell blocks obtained by various methods: cellient, formalin and thrombin. *Acta Cytol.* 2012; **56**; 289–296.
27. Hanley KZ, Birdsong GG, Cohen C, Siddiqui MT. Immunohistochemical detection of estrogen receptor, progesterone receptor, and human epidermal growth factor receptor 2 expression in breast carcinomas: comparison on cell block, needle-core, and tissue block preparations. *Cancer* 2009; **117**; 279–288.
28. FEDERA. Human tissue and medical research: Code of Conduct for responsible use. 2011. Available at: https://www.federa.org/codes-conduct (accessed 30 September 2019).
29. Evans M, O'Sullivan B, Hughes F *et al*. The clinicopathological and molecular associations of PD-L1 expression in non-small cell lung cancer: analysis of a series of 10,005 cases tested with the 22C3 assay. *Pathol. Oncol. Res.* 2020; **26**; 79–89.
30. Jin Y, Shen X, Pan Y *et al*. Correlation between PD-L1 expression and clinicopathological characteristics of non-small cell lung cancer: a real-world study of a large Chinese cohort. *J. Thorac. Dis.* 2019; **11**; 4591–4601.
31. Fitzgibbons PL, Bradley LA, Fatheree LA *et al*. Principles of analytic validation of immunohistochemical assays: guideline from the College of American Pathologists Pathology and Laboratory Quality Center. *Arch. Pathol. Lab. Med.* 2014; **138**; 1432–1443.
32. McHugh ML. Interrater reliability: the kappa statistic. *Biochem. Med. (Zagreb)* 2012; **22**; 276–282.
33. Koo TK, Li MY. A guideline of selecting and reporting intraclass correlation coefficients for reliability research. *J. Chiropr. Med.* 2016; **15**; 155–163.
34. Wang H, Agulnik J, Kasymjanova G *et al*. Cytology cell blocks are suitable for immunohistochemical testing for PD-L1 in lung cancer. *Ann. Oncol.* 2018; **29**; 1417–1422.
35. Gosney JR, Haragan A, Chadwick C *et al*. Programmed death ligand 1 expression in EBUS aspirates of non-small cell lung cancer: is interpretation affected by type of fixation? *Cancer Cytopathol.* 2020; **128**; 100–106.
36. Jain D, Sukumar S, Mohan A, Iyer VK. Programmed death-ligand 1 immunoexpression in matched biopsy and liquid-based cytology samples of advanced stage non-small cell lung carcinomas. *Cytopathology* 2018; **29**; 550–557.
37. Lloyd IE, Zhou W, Witt BL, Chadwick BE. Characterization of PD-L1 immunohistochemical expression in cell blocks with different specimen fixation and processing methods. *Appl. Immunohistochem. Mol. Morphol.* 2019; **27**; 107–113.
38. Giraldo N, Taube J. PD-L1 and other immunological diagnosis tools. In Zitvogel L, Kroemer G eds. *Oncoimmunology: a practical guide for cancer immunotherapy*. Cham, Switzerland: Springer International Publishing, 2017; 375–377.
39. Munari E, Zamboni G, Sighele G *et al*. Expression of programmed cell death ligand 1 in non-small cell lung cancer: comparison between cytologic smears, core biopsies, and whole sections using the SP263 assay. *Cancer Cytopathol.* 2019; **127**; 52–61.

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40. Thunnissen E, de Langen AJ, Smit EF. PD-L1 IHC in NSCLC with a global and methodological perspective. *Lung Cancer* 2017; **113**; 102–105.
41. Kruger AM, Stevens MW, Kerley KJ, Carter CD. Comparison of the Cellient™ automated cell block system and agar cell block method. *Cytopathology* 2014; **25**; 381–388.
42. Schmitt F, Cochand-Priollet B, Toetsch M, Davidson B, Bondi A, Vielh P. Immunocytochemistry in Europe: results of the European Federation of Cytology Societies (EFCS) inquiry. *Cytopathology* 2011; **22**; 238–242.
43. Fowler LJ, Lachar WA. Application of immunohistochemistry to cytology. *Arch. Pathol. Lab. Med.* 2008; **132**; 373–383.
44. Rekhtman N, Buonocore DJ, Rudomina D *et al.* Novel modification of HistoGel-based cell block preparation method: improved sufficiency for molecular studies. *Arch. Pathol. Lab. Med.* 2018; **142**; 529–535.
45. Dako Agilent Pathology Solutions. PD-L1 IHC 22C3 pharmDx is CE-IVD-marked for in vitro diagnostic use. Available at: https://www.agilent.com/cs/library/usermanuals/public/29171_22C3-ihc-pharmdx-interpretation-manual-eu.pdf (accessed 20 November 2019).
46. US Food and Drug Administration: Dako PD-L1 IHC 22C3 pharmDx. 2015. Available at: https://www.accessdata.fda.gov/cdrh_docs/pdf15/P150013c.pdf (accessed 20 November 2019).
47. Ventana Medical Systems, Inc: VENTANA PD-L1 (SP263) Assay. 2019. Available at: https://diagnostics.roche.com/global/en/products/tests/ventana-pd-l1-_sp263-assay2.html (accessed 20 November 2019).

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Concordance of programmed death-ligand 1 (PD-L1) positivity between SP263 and the 22C3 laboratory-developed test for specimens fixed in formalin and in Fixcyt, with two different cut-offs to determine PD-L1 positivity (≥1% and ≥50%).

Figure S1. The programmed death-ligand 1 (PD-L1) tumour proportion score (TPS) in three categories for material immunostained by use of the SP263 standardised assay and the 22C3 laboratory-developed test per case.

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