Lung Cancer 134 (2019) 233–237

Contents lists available at ScienceDirect

Lung Cancer

journal homepage: www.elsevier.com/locate/lungcan

Novel multiplex droplet digital PCR assay for scoring PD-L1 in non-small cell lung cancer biopsy specimens

Amanda Vannitamby[a], Shona Hendry[b], Louis Irving[c], Daniel Steinfort[c], Steven Bozinovski[a,*]

[a] School of Health & Biomedical Sciences, RMIT University, Bundoora, Victoria, Australia
[b] Department of Anatomical Pathology, St Vincent's Hospital, Melbourne, Victoria, Australia
[c] Department of Respiratory Medicine, Royal Melbourne Hospital, Melbourne, Victoria, Australia

ARTICLE INFO

Keywords:
Lung cancer
Immunotherapy
Non-small cell lung cancer
PD-L1

ABSTRACT

Objectives: Immune checkpoint inhibitors have become integrated into the clinical management of non-small cell lung cancer (NSCLC). Using RTqPCR, we have previously identified a gene expression panel that detected presence of malignant cells (MMP9:TIMP3 ratio) and quantified PD-L1 transcript levels in small biopsy specimens. However, RTqPCR has diagnostic limitations as it does not generate absolute copy number and is not readily multiplexed. To address this, we have developed a multiplex droplet digital PCR (ddPCR) assay.

Materials and methods: Biopsies obtained from NSCLC patients (n = 48 adenocarcinoma and n = 40 squamous cell carcinoma) and control lung biopsy specimens (n = 20) were analysed. Absolute MMP9, TIMP3 and PD-L1 transcript copy numbers were determined within a single assay by multiplex ddPCR using Taqman primers and the QX200 Droplet Digital PCR System.

Results and conclusions: Using our optimised triplex ddPCR assay, the MMP9:TIMP3 ratio was significantly elevated in NSCLC biopsies and using a cut-off of > 0.028, was 99% (95% CI; 80.5-94.5) sensitive and 80% specific for identifying malignant biopsies. The PD-L1:TIMP3 ratio significantly associated with PD-L1 tumour cell immunohistochemistry staining ($r = 0.539$, $p < 0.0001$) and was significantly higher in biopsies with > 50% PD-L1 tumour cell staining ($p < 0.0001$). In summary, a major advantage of our workflow is that it can accurately quantify PD-L1 tumour levels and provide sufficient nucleic acid for screening additional targetable mutations such as EGFR, ALK and ROS1 from a single small biopsy, thereby potentially avoiding the need for re-biopsy. Future studies will need to determine diagnostic ddPCR values that are predictive of clinical response to PD-1/PD-L1 immunotherapy.

1. Introduction

Immune checkpoint inhibitors that target programmed cell death 1 (PD-1) or programmed cell death-ligand 1 (PD-L1) are currently changing the approach to treatment of non-small cell lung cancer (NSCLC) patients. The Ventana PD-L1 (SP263) immunohistochemical assay is an FDA approved test for the detection of PD-L1 expression in formalin fixed, paraffin embedded (FFPE) specimens. Demonstration of high levels of PD-L1 expression by immunohistochemistry is associated with improved response rates in NSCLC patients treated with PD-1 inhibitors (Keynote-024, Keynote-010, Checkmate 017, Checkmate-057). Pembrolizumab is now approved as first line therapy for NSCLC patients with PD-L1 expression in > 50% of tumour cells, and as second line therapy for those demonstrating PD-L1 expression > 1% [1].

The current evaluation of PD-L1 expression is dependent on availability of FFPE tumour specimens. When available, FFPE tumour slides will firstly be used to evaluate presence of malignant cells, identify histological type of NSCLC and if warranted, screen for specific EGFR mutations and ALK and ROS1 rearrangements that are predictive of clinical response to tyrosine kinase inhibitors [2]. Hence, the assessment of PD-L1 expression is dependent on how much FFPE tumour tissue is left and the integrity of this fixed tissue specimen. We have recently taken an alternative approach to determine PD-L1 expression in small biopsy specimens using quantitative reverse transcription PCR (RTqPCR) [3]. Utilising minimally-invasive bronchoscopy and archived biopsy specimens, we identified the MMP9:TIMP3 mRNA ratio as a molecular marker for presence of malignant cells in NSCLC [3]. We also quantified PD-L1 gene expression by RTqPCR and demonstrated excellent concordance with the Ventana PD-L1 (SP263) Assay [3].

In this study, we have significantly advanced our approach by

* Corresponding author at: School of Health & Biomedical Sciences, RMIT University, Bundoora, VIC 3083, Australia.
E-mail address: steven.bozinovski@rmit.edu.au (S. Bozinovski).

https://doi.org/10.1016/j.lungcan.2019.06.029
Received 14 April 2019; Received in revised form 4 June 2019; Accepted 29 June 2019

developing a droplet digital PCR (ddPCR) assay to accurately and rapidly assess multiple biomarkers within the same specimen. ddPCR is an emerging molecular technique with increased sensitivity to detect driver mutations such as EGFR activating mutations. It has been shown to accurately detect and quantify EGFR variants in both tissue biopsies and plasma with greater sensitivity than amplification-refractory mutation system (ARMS) and may be more useful in monitoring disease progression [4]. ddPCR is also suitable for higher order multiplexing and is particularly sensitive at detecting low abundant variants [5]. Furthermore, ddPCR can address some significant limitations associated with RTqPCR, as it allows for absolute quantification of mRNA copy number, is less reliant on suitability of housekeeping genes and does not need to be normalised to a control specimen. We have specifically developed a multiplex ddPCR assay incorporating MMP9, TIMP3 and PD-L1 that generated absolute cut-off values, accurately identified presence of malignant cells in small NSCLC biopsies and quantified PD-L1 expression in a single assay.

2. Materials and methods

2.1. Patient characteristics

Matching snap frozen and FFPE specimens were provided by the Victorian Cancer Biobank with ethics approval (Ethics ID: SEHAPP 09–17) as previously detailed [3]. The specimens were obtained from surgically resected tumour tissue from patients with stage IA-IIIA NSCLC (n = 88) at the Royal Melbourne Hospital pathology department and included adenocarcinoma (n = 48) and squamous cell carcinoma (n = 40) subtypes. A 1–2 cm sample of tumour tissue excess to diagnostic requirements was obtained from the fresh resection specimen and bisected with one piece snap frozen and one piece formalin fixed and paraffin embedded as per standard laboratory protocols. Control tissue resection specimens (n = 20) were obtained from patients without malignant disease (n = 10) and adjacent tumour-free region of adenocarcinoma patients (n = 10), as previously described [3].

2.2. Direct quantification of gene expression by multiplex ddPCR

Total RNA from biopsies was isolated using the Allprep DNA/RNA/miRNA universal kit (Qiagen, Hilden, Germany) and converted to cDNA, as previously described [3]. The triplex ddPCR reaction used TaqMan primer/probe sets for each target sequence at a final concentration of 900 nM primer and 250 nM probe (Thermofisher MA, US). The MMP9 primer/probe (Hs00957562_m1) was used at a 1:1 mixture of VIC and FAM label. The TIMP3 primer/probe (Hs00165949_m1) was exclusively FAM labelled and the PD-L1 primer/probe (Hs01125301_m1) was exclusively VIC labelled. A 20 µl reaction mix of input cDNA (1–25 ng), ddPCR Supermix (Bio-Rad laboratories, CA, US) and all three Taqman primer/probes was combined and partitioned into 20,000 droplets using the QX200 Droplet Generator (Bio-Rad Laboratories, CA, US). Each reaction was transferred to 96-well PCR plate and endpoint PCR was performed using the C1000 Touch 96-deep well Thermal Cycler (Bio-Rad Laboratories, CA, US) at a ramp rate of 2 °C/s under the following conditions: 95 °C for 10 min., 40 cycles of 94 °C for 30 s and 60 °C for 1 min, followed by 98 °C for 10 min. Upon completion, the plate was read using the QX200 Droplet Reader (Bio-Rad Laboratories, CA, US). Absolute transcript levels were assessed using the QuantaSoft Analysis Pro software v 1.0 (Bio-Rad Laboratories, CA, US) amd the two-dimensional (2D) amplification plots were produced on the QuantaSoft Analysis Pro Software v 1.0 (Bio-Rad Laboratories, CA, US).

2.3. Scoring of PD-L1 immunohistochemical staining

FFPE tissue sections at 5 µm thickness were prepared from resected NSCLC tumour tissue adjacent to the frozen tissue biopsy specimen (n = 88). PD-L1 immunohistochemical scoring was performed by the Anatomical Pathology Laboratory at the Royal Melbourne Hospital using the human PD-L1 (SP263 CE IVD) rabbit monoclonal antibody (Ventana) and the Benchmark Ultra automatic staining instrument (Ventana, Tuscan, AZ, USA), as previously described [3]. The scoring of PD-L1 tumour and immune cell staining was blindly performed by an experienced pathologist [6].

2.4. Statistical analysis

Analysis was performed using Graphpad prism 7.02 (Graphpad Software Inc, San Diego, CA, including Spearman correlation coefficient, Mann-Whitney two-tailed t-tests between unpaired groups and paired analysis using the Wilcoxon signed rank test. The Kruskall Wallist test was performed to compare more than two groups. A Receiver Operating Characteristic (ROC) curve was generated and the area of the curve (AUC) was calculated to assess the accuracy of the MMP9:TIMP3 and PD-L1:TIMP3 ratio.

3. Results

3.1. The MMP9:TIMP3 ratio detects biopsies with malignant cells

The feasibility of measuring MMP9, TIMP3 and PD-L1 transcript levels using a triplex ddPCR assay was firstly assessed. A representative 2D plot shows the generation of seven distinct clusters using a combination of FAM and VIC labelled probes for the quantification of the three target sequences (Fig. 1A). FAM labelled TIMP3 is identified as a single positive cluster in channel 1 (T_A, burgundy) while VIC labelled PD-L1 is identified as a single-positive cluster in Channel 2 (T_B, purple). Using a 1:1 mixture of FAM and VIC labelled probes, MMP9 is identified as a single positive cluster in both channels (T_C, yellow). The generation of double-positive (T_{AB}, T_{AC}, T_{BC}) and triple-positive (T_{ABC}) clusters demonstrates the detection of 2 or more target sequences within the same droplet. Using this triplex assay, the ratio of MMP9:TIMP3 was significantly increased in the NSCLC specimens relative to control biopsies (Fig. 1B, 43.7-fold increase, $p < 0.0001$). The comparison between matched adjacent tumour-free control and NSCLC biopsies also identified a significant increase in tumour biopsies (Fig. 1C, 118.2-fold increase, $p < 0.01$). ROC curve analysis of the MMP9:TIMP3 ratio generated and AUC value of 0.945 (Fig. 1D, 95%CI; 0.88–1.0, $p < 0.0001$), which is consistent our previously published RTqPCR data [3]. A cut-off value of > 0.028 generated a sensitivity of 98.9% and specificity 80%, in discriminating malignant and control biopsies, demonstrating excellent diagnostic performance using our triplex assay.

3.2. The PD-L1:TIMP3 ratio identifies PD-L1 positive tumours

Assessment of PD-L1 by immunohistochemistry demonstrated that 52% and 50% of the tumours showed no tumour cell PD-L1 staining in adenocarcinoma and SCC respectively (Fig. 2A). There were proportionally more PDL1-high tumours based on > 50% tumour cell staining cut-off in SCC (17%, 7/40) than in adenocarcinoma (6%, 3/48). The degree of PD-L1 immune cell staining was also significantly higher in SCC when compared to adenocarcinoma (Fig. 2B). PD-L1 copy number was next expressed as a ratio relative to TIMP3 copy number using the same triplex ddPCR assay. Consistent with increased PD-L1 immunoreactivity in SCC, the PD-L1:TIMP3 ratio was only significantly increased in SCC relative to the control biopsy specimens (Fig. 2C). A positive association was observed between the PD-L1:TIMP3 ratio and PD-L1 tumour cell IHC staining performed on matching NSCLC biopsies (Fig. 2D; $r = 0.546$, $p < 0.0001$). In contrast, there was no significant association between the PD-L1:TIMP3 ratio and PD-L1 immune cell IHC staining (Fig. 2E; $r = 0.122$, $p = 0.259$). The ratio of PD-L1:TIMP3 was significantly higher in NSCLC biopsies that were graded as > 1% PD-L1

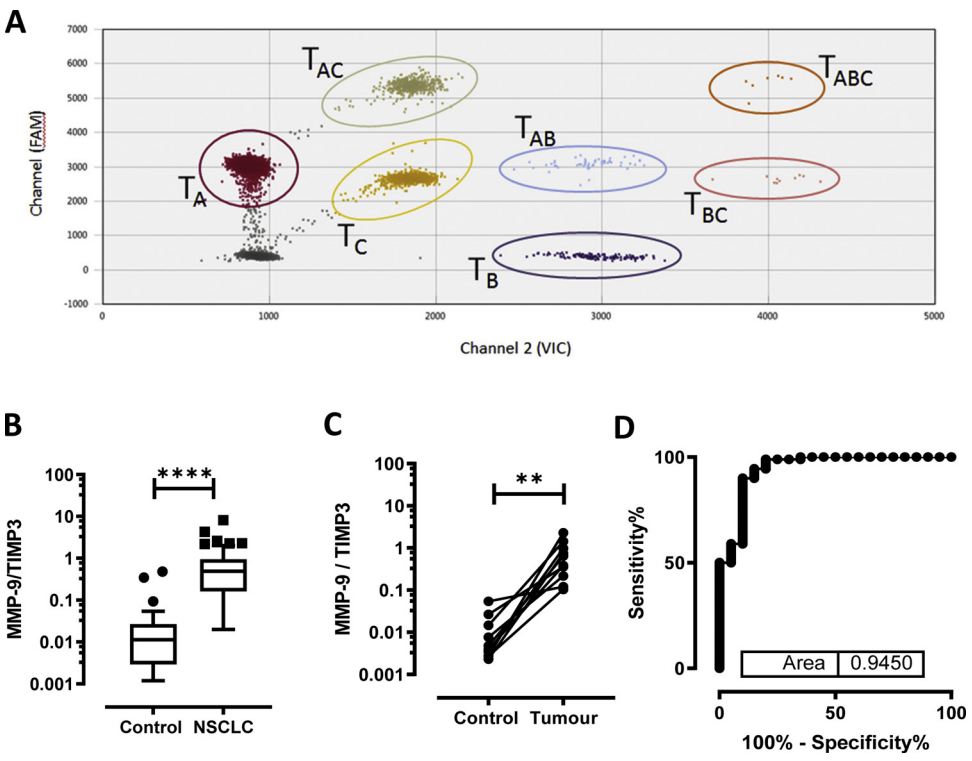

Fig. 1. Development of the triplex ddPCR assay for NSCLC diagnosis.
A triplex ddPCR reaction was performed for direct quantification of MMP9, TIMP-3 and PD-L1 (n = 108) simultaneously. (A) Displayed is a representative image of a 2D amplification plot showing 7 distinct clusters with each Target sequence (T) identified as either a single-positive, double-positive or triple-positive cluster. FAM-labelled TIMP-3 is identified in Channel 1 (T_A; burgundy) and VIC-labelled PD-L1 is identified in Channel 2 (T_B; purple). The third target sequence, MMP9, derived from a 1:1 mixture of FAM- and VIC- labelled probe is identified as a single positive cluster in both channels (T_C; yellow). Included in the final analysis are the double and triple positives for each target combination (T_{AB}; beige, T_{BC}; pink, T_{AC}; blue, T_{ABC}; orange). Negative droplets are shown in grey. The ratio of MMP9:TIMP3 obtained from the triplex assay was significantly elevated in the NSCLC specimens relative to the unmatched controls (B, ****$p < 0.0001$, Mann-Whitney t test) and matched controls (C, **$p < 0.01$). A ROC curve analysis of the MMP9:TIMP3 ratio produced an AUC value of 0.945 (D, 95%CI; 0.88–1.0, ****$p < 0.0001$) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

positive tumour cell staining (Fig. 2F, 3.4-fold increase, $p < 0.0001$). Using a higher PD-L1 tumour cell staining threshold, the ratio of PD-L1:TIMP3 was also significantly increased in tumours with > 50% staining (Fig. 2G, 12.7-fold increase, $p < 0.0001$). ROC curve analysis of the PD-L1:TIMP3 ratio generated an AUC value of 0.961 (Fig. 2H, 95%CI; 0.90–1.0) for the SCC specimens and an AUC value of 0.926 (Fig. 2H, 95%CI; 0.80–1.0) for the adenocarcinoma specimens. Using a ddPCR cut off > 0.104, the PDL1:TIMP3 ratio was 90% sensitive and 89% specific in identifying NSCLC tumours with > 50% PD-L1 positive IHC staining.

4. Discussion

As more targeted agents and immunotherapeutic options become available for the treatment of cancer, the number of essential predictive biomarkers also increases, putting pressure on pathologists and oncologists to make the best use of often limited tissue samples to avoid repeated invasive procedures. Some specimen types are particularly problematic, for example endobronchial biopsies are often small and crushed making PD-L1 IHC interpretation challenging, while cytology specimens often contain insufficient cells in the cell block for reliable PD-L1 IHC. A major advantage of our ddPCR workflow is the ability to quantify PD-L1 tumour expression and provide sufficient nucleic acid to test for additional targetable mutations in EGFR, ALK and ROS1 from a single small specimen. We have previously demonstrated that a single EBUS bronchoscopy specimen is sufficient to quantify multiple target genes [3], thereby potentially avoiding the need for re-biopsy. Furthermore, our workflow could be easily integrated into current practices as EBUS specimens are routinely collected fresh before fixatives are added. Our fixative approach will specifically stabilise and generate high quality and quantity of nucleic acid for multiple molecular tests. Multiplexing ddPCR simplified the quantification of our target transcripts by directly and simultaneously generating absolute copy number for multiple transcripts. When using an MMP9:TIMP3 ratio cut-off of > 0.028, our triplex ddPCR assay identified malignant specimens with very high sensitivity and specificity, and hence can be used to determine whether the specimen is suitable for additional molecular tests including PD-L1 quantification.

PD-L1 IHC is acknowledged to be an imperfect biomarker, and the three most concordant commercially available IHC assays have an agreement of approximately 85% [7]. In our study, a similar level of concordance was observed between PD-L1 tumour cell IHC and PD-L1 levels determined by ddPCR, where 11/88 tumour biopsies identified as PD-L1 high by ddPCR were scored negative by IHC based on the < 50% IHC score. These specimens included biopsies with increased PD-L1 tumour cell staining that fell below the 50% threshold (range 5–40%) and biopsies with elevated immune cells staining and prominent alveolar macrophage infiltration. Alveolar macrophage expression of PD-L1 is a known confounder that particularly affects adenocarcinomas compared to SCCs, due to tumour architecture. The inability to discriminate the cell type expressing PD-L1 is acknowledged as a limitation of this testing method, however the impact of this limitation on the predictive ability of the test remains to be fully elucidated. It could be argued that assessment of clinical response to anti-PD-1 treatment is really the best “gold standard” against which to compare different PD-L1 biomarker methodologies. Future studies are needed to determine diagnostic ddPCR values that are predictive of clinical response to PD-1/PDL1 immunotherapy before clinical implementation of our assay. What has also recently become apparent is that PD-L1 expression on tumour and infiltrating immune cells has non-redundant roles in regulating anti-cancer immunity, where PD-L1 expression on both cell types is important for predicting best response to atezolizumab in NSCLC [8]. Our data demonstrates that whilst PD-L1 tumour expression is the dominant driver of increased PD-L1 transcript levels in NSCLC, immune cell infiltration can also contribute to absolute PD-L1 mRNA copy number.

Another important advantage is that our triplex ddPCR assay can be automated, performed within 24–48 h of specimen collection and only requires standard ddPCR consumable reagents. The analysis can be readily adapted into an automated, quantitative process to remove potential inter-assay and inter-observer variation in the scoring of PD-L1 staining on FFPE sections. A limitation of our study is that remains to

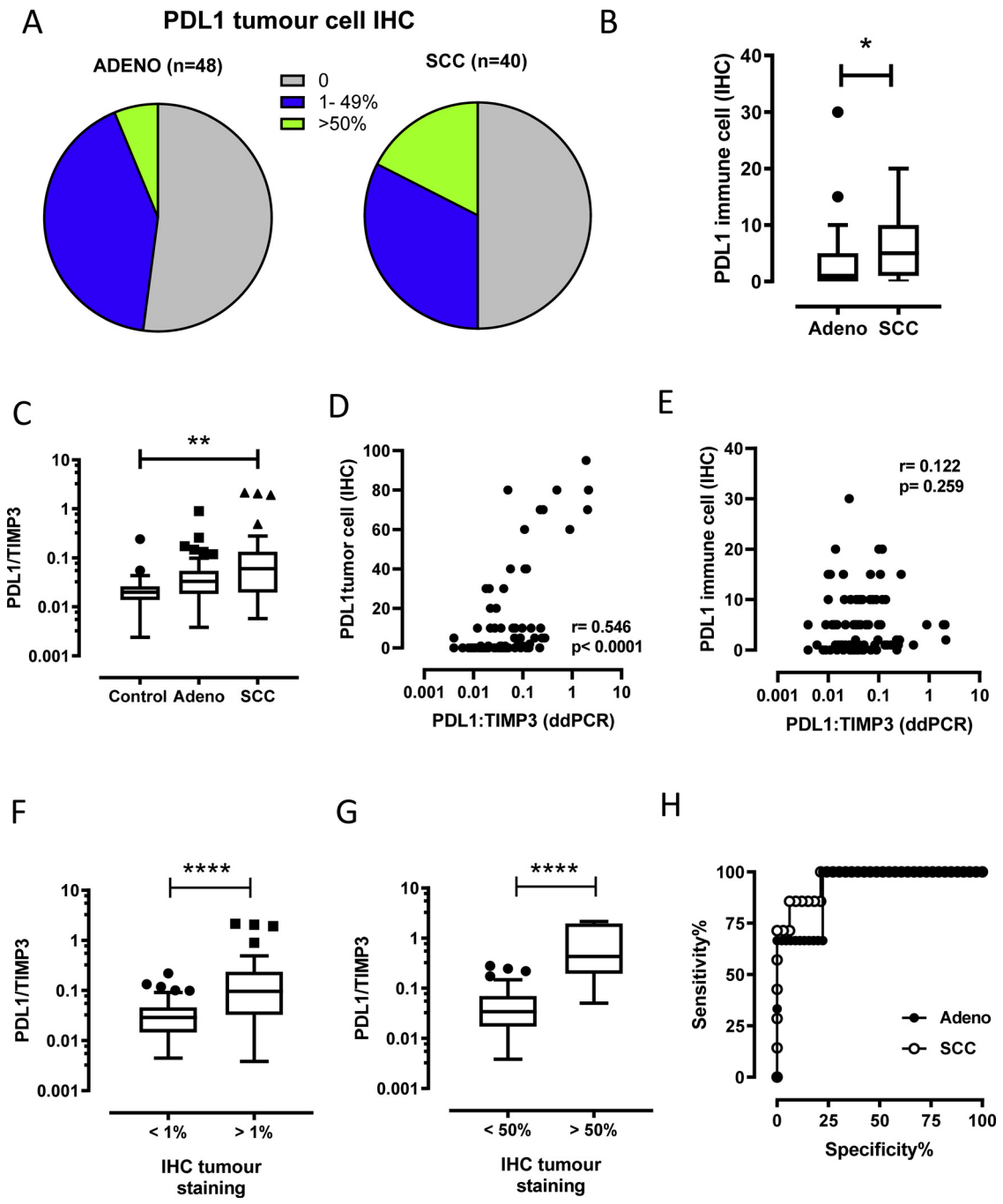

Fig. 2. Utilising the triplex ddPCR assay for determining PD-L1 status.
(A) PD-L1 tumour cell IHC staining of NSCLC blocks (n = 88) demonstrates a differential staining pattern where there was a greater proportion of PD-L1 high (> 50%) tumours in SCC (17%) compared to the adenocarcinoma blocks (6%). (B) Scoring of PD-L1 immune cell IHC staining using the same biopsies revealed a significant increase in SCC (*$p < 0.05$). (C) Using our triplex ddPCR assay, the ratio of PD-L1:TIMP3 was significantly elevated in SCC but not adenocarcinoma tumour specimens when compared to control biopsies (* $p < 0.05$). Spearman analysis demonstrates that the PD-L1:TIMP3 ratio significantly correlates with (D) PD-L1 tumour cell IHC staining but not (E) PD-L1 immune cell IHC staining. The PD-L1:TIMP3 ratio was significantly increased in (F) NSCLC biopsies with > 1% PD-L1 tumour cell IHC staining and in (G) NSCLC biopsies > 50% PD-L1 tumour cell IHC staining (****$p < 0.0001$). (H) The PDL1:TIMP3 ratio discriminated PD-L1 low (< 50% by IHC) from PD-L1 high (> 50% by IHC) specimens with ROC curve analysis generating an AUC value of 0.961 (95%CI; 0.90–1.0) for the SCC specimens and an AUC value of 0.926 (95%CI; 0.8–1.0) for the adenocarcinoma specimens.

be established whether small EBUS-TBNA specimens would be adequate for our multiplex ddPCR workflow. However, we have recently shown that a single pass snap frozen EBUS-TBNA specimen yielded highly intact DNA (4 µg), which is well above the minimum threshold for ddPCR or targeted sequencing [9]. In summary, we propose the following workflow where our triplex ddPCR assay can complement current gold standards. Following tumour cell confirmation by rapid on-site evaluation, an EBUS or other endoscopic specimen comprising of 3 or more passes should be obtained and formalin-fixed paraffin embedded for lung cancer diagnosis and staging as previously described [10]. A final single pass EBUS-directed specimen from the same site should then be collected and either snap frozen or stored in a nucleic acid stabilisation buffer, which will provide a high yield of unfixed nucleic acid for evaluating PD-L1 levels by ddPCR and allow for

additional mutation detection.

Funding

National Health and Medical Research Council of Australia (NHMRC).

Author declaration

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all of us.

We confirm that we have given due consideration to the protection of intellectual property associated with this work and that there are no impediments to publication, including the timing of publication, with respect to intellectual property. In so doing we confirm that we have followed the regulations of our institutions concerning intellectual property.

We further confirm that any aspect of the work covered in this manuscript that has involved human patients has been conducted with the ethical approval of all relevant bodies and that such approvals are acknowledged within the manuscript.

References

[1] L. Pai-Scherf, G.M. Blumenthal, H. Li, S. Subramaniam, P.S. Mishra-Kalyani, K. He, H. Zhao, J. Yu, M. Paciga, K.B. Goldberg, A.E. McKee, P. Keegan, R. Pazdur, FDA approval summary: pembrolizumab for treatment of metastatic non-small cell lung cancer: first-line therapy and beyond, Oncologist 22 (11) (2017) 1392–1399.

[2] M. Dietel, L. Bubendorf, A.M. Dingemans, C. Dooms, G. Elmberger, R.C. Garcia, K.M. Kerr, E. Lim, F. Lopez-Rios, E. Thunnissen, P.E. Van Schil, M. von Laffert, Diagnostic procedures for non-small-cell lung cancer (NSCLC): recommendations of the European Expert Group, Thorax 71 (2) (2016) 177–184.

[3] A. Vannitamby, S. Hendry, T. Makadia, J. Danks, J. Slavin, L. Irving, D. Steinfort, S. Bozinovski, A novel approach to detect programed death ligand 1 (PD-L1) status and multiple tumor mutations using a single non-small-cell lung cancer (NSCLC) bronchoscopy specimen, J. Mol. Diagn. 21 (2) (2019) 186–197.

[4] T.K.F. Yung, K.C.A. Chan, T.S.K. Mok, J. Tong, K.-F. To, Y.M.D. Lo, Single-molecule detection of epidermal growth factor receptor mutations in plasma by microfluidics digital PCR in non–small cell lung cancer patients, Clin. Cancer Res. 15 (6) (2009) 2076–2084.

[5] A.S. Whale, J.F. Huggett, S. Tzonev, Fundamentals of multiplexing with digital PCR, Biomol. Detect. Quantif. 10 (2016) 15–23.

[6] S. Hendry, D.J. Byrne, G.M. Wright, R.J. Young, S. Sturrock, W.A. Cooper, S.B. Fox, Comparison of four PD-L1 immunohistochemical assays in lung cancer, J. Thorac. Oncol. 13 (3) (2018) 367–376.

[7] F.R. Hirsch, A. McElhinny, D. Stanforth, J. Ranger-Moore, M. Jansson, K. Kulangara, W. Richardson, P. Towne, D. Hanks, B. Vennapusa, A. Mistry, R. Kalamegham, S. Averbuch, J. Novotny, E. Rubin, K. Emancipator, I. McCaffery, J.A. Williams, J. Walker, J. Longshore, M.S. Tsao, K.M. Kerr, PD-L1 immunohistochemistry assays for lung cancer: results from phase 1 of the blueprint PD-L1 IHC assay comparison project, J. Thorac. Oncol. 12 (2) (2017) 208–222.

[8] M. Kowanetz, W. Zou, S.N. Gettinger, H. Koeppen, M. Kockx, P. Schmid, E.E. Kadel 3rd, I. Wistuba, J. Chaft, N.A. Rizvi, D.R. Spigel, A. Spira, F.R. Hirsch, V. Cohen, D. Smith, Z. Boyd, N. Miley, S. Flynn, V. Leveque, D.S. Shames, M. Ballinger, S. Mocci, G. Shankar, R. Funke, G. Hampton, A. Sandler, L. Amler, I. Mellman, D.S. Chen, P.S. Hegde, Differential regulation of PD-L1 expression by immune and tumor cells in NSCLC and the response to treatment with atezolizumab (anti-PD-L1), Proc. Natl. Acad. Sci. U. S. A. 115 (43) (2018) E10119–E10126.

[9] T.L. Leong, M. Christie, S. Kranz, K. Pham, A. Hsu, L.B. Irving, M.L. Asselin-Labat, D.P. Steinfort, Evaluating the genomic yield of a single endobronchial ultrasound-guided transbronchial needle aspiration in lung cancer: meeting the challenge of doing more with less, Clin. Lung Cancer 18 (6) (2017) e467–e472.

[10] D.P. Steinfort, P.A. Russell, A. Tsui, G. White, G. Wright, L.B. Irving, Interobserver agreement in determining non-small cell lung cancer subtype in specimens acquired by EBUS-TBNA, Eur. Respir. J. 40 (3) (2012) 699–705.