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Automated chromogenic multiplexed immunohistochemistry assay for diagnosis and predictive biomarker testing in non-small cell lung cancer

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ABSTRACT

Objectives: The current challenge in the management of non-small cell lung cancer (NSCLC) in pathology laboratories is to combine immunohistochemistry (IHC) and molecular approaches on increasingly smaller biopsies and the need to reserve a fair amount of tumor material for molecular analyses with increasingly larger panels. The latest lung cancer classification, especially in the setting of poorly differentiated tumors, requires an IHC workup to allow for accurate diagnosis and also to preserve as much tissue as possible for molecular testing. Thus, it is recommended to reduce use of the term NSCLC not otherwise specified as much as possible and classify tumors according to their specific histologic subtype. This implies limiting the number of tissue slides despite the existence of specific and sensitive biomarkers (ALK, ROS1, BRAF V600E, PD-L1) and the obligation to distinguish lung adenocarcinoma (TTF-1 positive) from squamous cell carcinoma (p40 positive).
Materials and Methods: Samples from 18 patients with NSCLC, previously characterized for histologic and genomic/immune features, were included. Two multiplexed IHC assays were developed, for diagnosis and immunophenotyping including TTF1, p40, PD-L1, and pan-Keratin antibodies, and for molecular profiling panel including ALK, ROS1 and BRAF V600E antibodies.
Results: We developed two sensitive multiplexed IHC assays to comprehensively characterize major NSCLC histotypes and FDA-cleared predictive biomarkers, without antigenicity loss, steric interference or increased cross-reactivity. The assays rely on standard antigen retrieval and automated staining protocols, limiting the need for validation strategies.
Conclusion: Our multiplexed IHC approach provides a unique sample-sparing tool to characterize limited tissue samples in lung oncology and making it an alternative method in the clinical setting for therapeutic decision making of advanced NSCLC, provided that validation in a larger population is performed.

1. Introduction

Current standards of care of non-small cell lung cancer (NSCLC) patients assign therapeutic decisions on the basis of specific histologic and genomic/immune-based characteristics of the patient's tumor [1]. NSCLC must be classified into different histotypes [*e.g.*, lung adenocarcinoma (LADC) *versus* lung squamous cell carcinoma (LSCC)] as this determines eligibility for biomarker testing and then therapeutic strategies [2]. The current list of indispensable biomarkers associated to approved therapies worldwide include *EGFR* mutations, *ALK* and *ROS1* rearrangements, the *BRAF* V600E mutation in LADC and PD-L1 expression in tumor cells in both LADC and LSCC [3]. However, as the amount of histologic and genomic/immune-based information required has grown recently, analysis of tumor tissue often rests on small tissue samples [4].

To address the increase in the essential clinical need for high-output diagnostic and predictive biomarker testing in NSCLC, we developed an automated brightfield multiplexed chromogen-based immunohistochemistry (mIHC) assay that could be readily integrated into the routine clinical setting.

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2. Methods

2.1. Tumor samples

For this study, formalin-fixed paraffin-embedded samples from 18 patients with NSCLC, previously characterized for histologic [LADC, n = 3; LSCC, n = 3; or lung adenosquamous carcinoma, LASC, n = 3) and genomic/immune features (*ALK*, n = 3; *ROS1*, n = 3; *BRAF* V600E, n = 3; PD-L1, n = 3), were obtained from the Hospital-Integrated Biobank (BB-0033-00025, Pasteur Hospital, Nice) after IRB approval and informed written consent.

2.2. Multiplex immunohistochemistry

Two mIHC assays were developed: *i)* the mIHC for diagnosis and immunophenotyping (MIDI) panel including TTF1, p40, PD-L1, and pan-Keratin antibodies, and *ii)* the mIHC for molecular profiling (MIMP) panel including ALK, ROS1 and BRAF V600E antibodies. The assays were performed on the Discovery Ultra autostainer (Ventana Medical Systems, Tucson, AZ), by sequentially applying unmodified primary antibodies (Abs) with specific Heat Deactivation steps in between for elution (Table 1) [5].

2.3. Interpretation of mIHC staining

mIHC tumor proportion score (mTPS) was calculated as the percentage of PD-L1–cells colored in Teal (defined as complete circumferential or partial cell membrane staining of tumor cells) relative to all tumor cells colored in Yellow present in the tumor sample. Any nuclear staining (Purple/Silver) in tumor cells stained in Yellow was considered positive for TTF1 and p40. Specimens were scored positive for ALK (Purple), ROS1 (DAB) and BRAF V600E (Teal) if strong cytoplasmic staining in any percentage of tumor cells was present. Interpretation was assessed blindly by three trained pathologists (MI, VH, and PH)

2.4. Statistical analysis

Statistical analyses to assess the inter-pathologist agreement of the mIHC stainings were performed on PD-L1 TPS scores as continuous variables (*e.g.* intra-class correlation coefficient) as well as on dichotomized variables as positive and negative (*e.g.* κ score) for TTF1, p40, ALK, ROS1, and BRAF V600E. κ scores were calculated to assess concordance between mIHC staining panels and single conventional IHC DAB staining for each marker.

3. Results

The staining pattern of individual DAB-stained slides was compared with the matched single colored chromogen slide and the mIHC slide (Fig. 1). Comparison of mIHC staining panels with single conventional IHC DAB staining showed equivalent sensitivity and specificity for histotypes and genomic/immune markers throughout the 18 NSCLC cases included in the study (κ scores = 1, respectively). In addition, κ scores for inter-pathologist agreement were 1 for all dichotomous markers. The intra-class correlation coefficient, used to measure the inter-pathologist agreement of PD-L1 mTPS score as continuous variable, was 100%.

The MIDI panel depicts TTF1 + LADC cells *versus* p40 + SCC cells, including PD-L1 + in both LADC and SCC cells, while the MIMP panel visualizes ALK + *versus* ROS1 + *versus BRAF* V600E + tumor cells.

All the markers showed comparable staining pattern at the subcellular location and similar percentage of positive cells between the two mIHC assays and single DAB slide. No cross-reactivity was observed

Table 1
Summary of technical conditions used for the two mIHC detection assays. (For interpretation of the references to color in this Table legend, the reader is referred to the web version of this article.)

	mIHC for Diagnosis and Immunophenotyping (MIDI) panel TTF1-p40-PD-L1-Keratin	mIHC for Molecular Profiling (MIMP) panel ALK-ROS1-BRAF V600E
Pretreatment	Cell Conditioning 1 (CC1, #950-500) 40 min 95°C	CC1 92 min 100°C
Inhibitor	Discovery Inhibitor (#760-4840) 4 min	Discovery Inhibitor (#760-4840) 4 min
Primary Ab#1	Anti-p40 mouse monoclonal Ab (BC28 clone, prediluted, #790-4950) 60 min	Anti-ROS1 rabbit monoclonal Ab (D4D6 clone, dilution 1:50, #3287, Cell Signaling) 60 min
Detection	OmniMap anti-mouse HRP (#760-4310) 16 min	Anti-Rabbit-HQ (#760-4815) 32 min + anti-HQ-HRP (#760-4820) 32 min
Amplification	No	AMP HQ 12 min
Chromogen	Silver kit (#760-227), 16 min	DAB Map Detection Kit (#760-124) 16 min
Elution	Cell Conditioning 2 (CC2, #950-123) 8 min 100°C	CC2 8 min 100°C
Primary Ab#2	Anti-PD-L1 rabbit monoclonal Ab (clone SP263, prediluted, #741-4905), 120 min	anti-ALK rabbit monoclonal Ab (D5F3 clone, prediluted, #790-4796) 60 min
Detection	Anti-Rabbit-HQ (#760-4815) 16 min + anti-HQ-HRP (#760-4820) 16 min	Anti-Rabbit-HQ (#760-4815) 20 min + anti-HQ-HRP (#760-4820) 20 min
Amplification	AMP HQ 12 min	AMP HQ 32 min
Chromogen	Discovery Teal-HRP (#760-247) 32 min	Purple Kit (#760-229) 40 min
Elution	CC2 8 min 100°C	CC2 8 min 100°C
Primary Ab#3	Anti-TTF1 (SP141) rabbit monoclonal Ab (#790-4756, prediluted) 60 min	Anti-BRAF V600E mouse monoclonal Ab (VE1 clone, prediluted, #790-4855) 40 min
Detection	OmniMap anti-rabbit HRP (# 760-4311) 16 min	Anti-Mouse HQ (#760-4814) 16 min + anti-HQ-HRP (#760-4820) 16 min
Amplification	No	AMP HQ 32 min
Chromogen	Purple Kit (#760-229) 32 min	Discovery Teal-HRP (#760-247) 32 min
Elution	CC2 8 min 100°C	
Pretreatment	Protease 3 (#760-2020), 4 min	
Primary Ab#4	anti-Pan Keratin mouse monoclonal Ab (clone AE1/AE3/PCK26, prediluted, #760-2135) 16 min	
Detection	UltraMap anti-mouse Alk Phos (#760-4312) 12 min	
Chromogen	Yellow kit (#760-239) 44 min	

Chromogens colors are shown in corresponding cells. The slides were colored using #Ventana reagents except when noted.