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Review Article

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Immunohistochemistry as Predictive Biomarkers in Non-Small Lung Cancer: A Review and Discussion

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Abstract

This is a discursive review of the role of immunohistochemical assays as predictive biomarkers in patients with non-squamous, non-small cell lung carcinoma.

Introduction

This review article examines the role of immunohistochemistry (IHC) for detection of biomarkers reported by cellular pathologists required for oncological treatment decisions in a setting of non-squamous, non-small cell lung cancers. Discussion is made for and against the use of IHC in this context and compares the utility of such an approach with other molecular testing methods. For the purposes of this manuscript, predictive biomarkers are defined as those used after a histopathological diagnosis has been made to inform targeted treatments. Predictive biomarkers with an underlying specific genetic alteration, such as point mutations in exons 18-21 of the EGFR gene or translocations effecting ALK1, are discussed.
PD-L1, which is overexpressed in tumours with a non-specific, multi-mutational profile (high tumour mutational burden) fall outside the scope of this paper.

ALK-1

Immunohistochemistry (IHC) is a technique that has been used in clinical cellular pathology diagnostics for decades to detect protein expression (1-4). The diagnostic utility of IHC is phenomenal and enhanced by its spatial context, low cost and rapid turnaround time (5). See figure 1.

Recent advances in diagnostic immunohistochemistry include multiplex assays that have developed as digital pathology has improved (6, 7). It is important that all immunohistochemical stains for use in treatment decisions uses standardised testing protocols, appropriate tissue fixation methods, tissue controls and antibody clones (8). Medical laboratory standards should also be inspected through participation in external quality assurance schemes and annual accreditation visits by UKAS (9, 10).

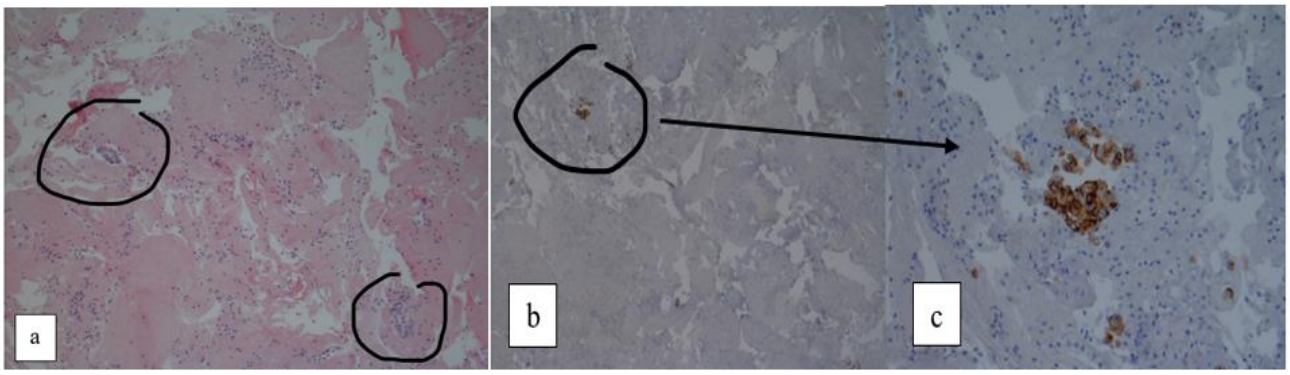

Figure 1: A pleural fluid cytology case of low cellularity as seen in the low-power view of an H&E section in 5a. Black circles indicate scanty malignant cell groups present. ALK-1 immunohistochemistry (5b, low-power view, and 5c, high-power view showing strong positive cytoplasmic staining in malignant cells and illustrating the utility of IHC in low cellularity specimens by giving a spatial context. Specimens with less than 100 malignant cells rarely give meaningful RNA sequencing results(11). The IHC findings in this case were corroborated by ALK-1 FISH using break-apart probes 31 days after the IHC was reported.

IHC for anaplastic lymphoma kinase (ALK-1) fusion protein has been used in lung cancer care for many years (12). It relies upon detection of *ALK* fusion oncogenic driver protein within the cytoplasm of malignant cells, the most common partner of which is *EML4* (13, 14). The fusion of *EML4* with *ALK* drives overexpression of the protein which is not normally expressed in lung tissue. Accurate detection of ALK-1 fusion protein requires use of a specific clone, D5F3, which uses a multiplex

linker technology signal to highlight low levels of the cytoplasmic protein to avoid reporting false negative cases (15). The D5F3 clone is classified as a companion diagnostic biomarker and has been studied in clinical trials alongside tyrosine kinase inhibitor drugs such as Crizotinib, Lorlatinib and Ceritinb. The D5F3 ALK-1 antibody clone is quite different in its sensitivity and specificity from other clones used in diagnostic haematopathology (16).

Most cellular pathology centres in the UK use ALK-1 IHC as a screening tool to detect the majority of negative NSCLC cases (96%) due to its high negative predictive value, cost and turnaround time (17). False negative and positive results can occur in a minority of cases, most in cytology preparations without sufficient levels of pre-analytical formalin fixation to comply with standard IHC protocols for antigen retrieval (18). In addition, one needs to exercise caution in interpreting findings in mucin-secreting tumours (19, 20). See figure 2 However, the Food and Drug Administration (FDA) in the US has recognised the D5F3 clone of ALK-1 antibody as of sufficiently high standard upon which to base decisions to treat in NSCLC without the need for molecular confirmation (21). Some suggest that use of RNA-based sequencing is a better use of small tissue biopsies where multiplex identification of a number of oncogenic fusion drivers can be detected with one test. However, this assertion does not take into account the large amount of tissue required for NGS nor the high failure rate (22, 23). Audit data from my practice suggests an RNA sequencing failure rate of 35% in NSCLC (24).

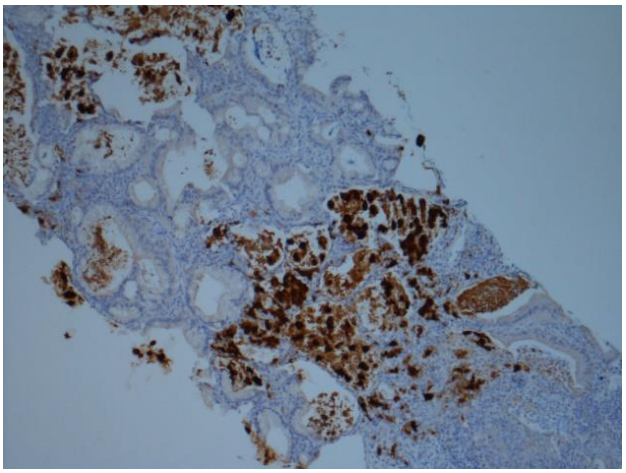

Figure 2: An example of ALK-1 IHC staining in a mucin-secreting NSCLC adenocarcinoma. There is extensive non-specific uptake in extra-cellular and luminal mucin. Cells of malignant gland have negative cytoplasm.

Furthermore, there is evidence that detection of the protein-drug target in tumour cells offers addition information about prognosis and drug response that cannot be gleaned from NGS or FISH. Patients with expression of ALK-1 in their tumours tend to have a higher response rate than those with ALK-1 IHC negative/ FISH or NGS rearrangement positive tumours (25, 26). This makes sense when one considers that the drug target is the tumour protein in malignant cells rather than the structural rearrangement in DNA.

ROS-1

Gene rearrangements giving rise to over-expression of ROS-1 protein (D4D6 companion diagnostic IHC clone) are seen in a small minority of non-squamous, NSCLC patients; Prevalence is in the region of 0.5-1% (12). Patients with ROS-1 rearrangements show sustained progression free survivals when treated TKIs (19). Similar pre-analytic conditions apply to ROS-1 immunohistochemistry as ALK-1 with reliance of FFPE tissue sections where tissue has been fixed in 10% neutral buffered formalin for between 6 and 72 hours. Tissue sections ideally should be cut fresh as the tissue slide stability for protein detection is reduced after around 3 months, similar to ALK-1 IHC (12). Fusion partners in *ROS-1* rearrangements are much more varied than *ALK-1* fusions and some authors suggest that the appearance and pattern of staining with ROS-1 IHC varies with fusion partner (27). Having said this, ROS-1 IHC positive cases usually show a diffuse cytoplasmic expression but can be weak and must be confirmed with orthogonal tests such as FISH or RNA-based NGS (28). See figure 3a and 3b. This is because type 2 pneumocytes in normal and reactive lung tissue may express low levels of ROS-1 cytoplasmic protein (12, 29). See figure 3c.

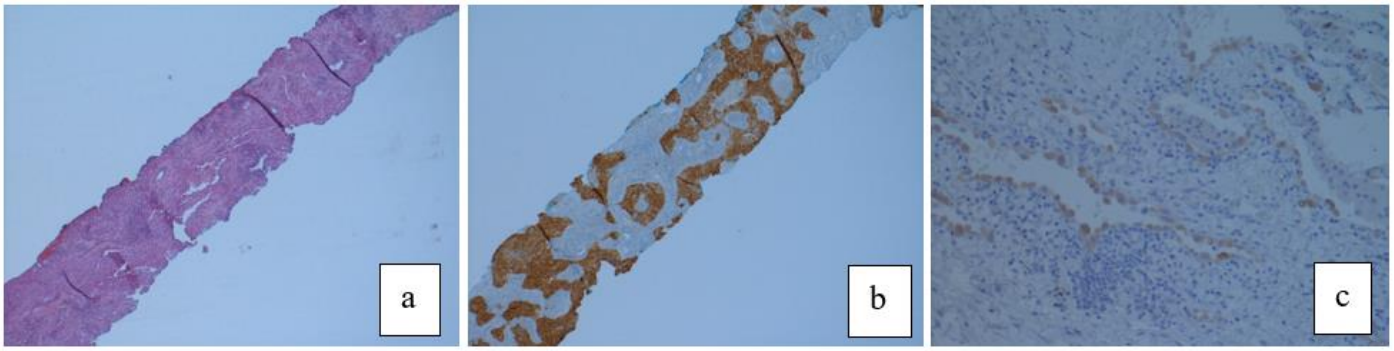

Figure 3: ROS-1 Immunohistochemistry. 7a) Illustrates the H&E appearances of a solid pattern, primary pulmonary adenocarcinoma. 7b) Illustrates the typical diffuse strong pattern of ROS-1 immunohistochemical staining see in ROS-1 fusion positive cases. Subsequent FISH failed to corroborate this finding. The patient was a life-long smoker and started immunotherapy but unfortunately died 5 months later. 7c) Background cytoplasmic expression in reactive type 2 pneumocytes lining alveolar spaces in human lung (high-power).

Furthermore, some studies have shown that *ERBB2*-mutated lung adenocarcinomas can express *ROS-1* and that false positive ROS-1 IHC is frequently seen in a setting of mucinous differentiation (30, 31). We use ROS-1 IHC in clinical practice in Swansea and have a higher reliance on FISH and or RNA-based sequencing for ultimate decision making regarding oncological therapy. We find it useful for screening our clear negative cases given the low incidence of ROS-1 gene rearrangements and its rapid turnaround time.

panTRK

The Neurotrophic Receptor Kinase (*NTRK*) genes code for membrane bound Trk receptors that link to the MAPK intra-cellular signalling pathway *(NTRK1*), Ras/ERK signalling pathway (*NTRK2*) and the PI3K/PKB pathway (*NTRK3*) upon binding of their respective ligands at the cell surface (32). Their activation leads to cell growth and proliferation stimulation, and promotes cell survival, invasion and angiogenesis (32), all molecular features involved in development of malignancy (33, 34). Fusions affecting *NTRK* genes 1 and 3, resulting in overexpression of pan-Trk receptors, have been documented in NSCLC (35). *NTRK 2* fusions are said to be very rare in NSCLC and appear to be concentrated in central nervous system lesions (36). Interestingly it has been shown that overexpression of TrkB in small cell neuroendocrine carcinoma is associated with a poorer prognosis also (37). Chetty and others indicate that use of pan-Trk immunohistochemistry is an attractive option for testing due to the low incidence of *NTRK* fusion events in NSLC (between 1-5%), rapid turnaround time, wide accessibility in cellular pathology laboratories, low cost and the high specificity of the antibody (32, 38-44). The utility of pan-Trk immunohistochemistry applies to all solid tumours in adults since Entrectinib and Larotrectinib (Trk inhibitors) were licensed for use in a tumour agnostic fashion by NICE (45-47). Both drugs have high response rate and give patients long term disease control in the presence of *NTRK* gene fusions (45, 48) but treatment with Trk inhibitors should only be commenced when standard chemotherapy and other targeted therapies have failed (46, 47).

Whilst many acknowledge the benefits of IHC, alternative methods of testing are becoming more widely available. A retrospective study of over 38,000 patient samples showed a DNA-based NGS panel to have an overall sensitivity of 81.1% and specificity of 99.9% (42). Furthermore, the detection of a fusion event in DNA does not give functional information regarding transcription status (49). RNA-based NGS is the preferred option for detection of fusion events as the intronic sequences in all 3 genes are large and are difficult to identify with bioinformatic pipelines(50). Assessing mRNA transcripts where introns have been spliced out makes the bioinformatic analysis simple and reliable for clinical reporting (11, 51). Analytical sensitivity was shown to be between 86.6 and 100% (n=15 and specificity 100% in a study of three RNA-based sequencing assays (52).

RNA-based sequencing for detection of all fusion events in NSCLC has been used in Wales since October 2021. A recent audit of a small cohort of my patient reports showed a failure rate of 35% (24). This figure reflects our experience in Swansea where our molecular diagnostic sequencing is conducted in a hub in Cardiff. This failure rate is in keeping with other centres in England using the centralised Genomic Laboratory Hubs (GLH) approach to testing (23, 53). The cause of RNA sequencing assay failure in our region cannot be explained by paucity of tissue as we refrain from sending samples with insufficient malignant tissue (11, 54). Rather the failure rate we experience is more likely a consequence of the fragility of the RNA molecule(55). The additional hydroxyl group on the ribose structure of RNA makes the molecule more vulnerable to hydrolysis In addition there are RNase degradation enzymes present throughout the environment, on human hands, in cellular pathology laboratories and work benches (56). Furthermore, formalin fixation is known to directly degrade RNA and can also interfere with the effectiveness of agents used in RNA extraction and library preparation (57-59). Cold ischaemic time, defined as the time a surgical or biopsy specimen sits waiting to be fixed in formalin, is also a major contributor to RNA breakdown and a pre-analytical step beyond the control of pathology laboratories (60, 61). And, finally, the increasing length of time an FFPE tissue block resides in archive, the more RNA will have degraded prior to testing (62). These factors might account for the failure rate of RNA-based NGS to detect gene fusion onco-drivers in NSCLC but may be ameliorated by use of the Idylla

GeneFusion assay which has a failure rate of 2% in comparison (24, 63).

RET

RET fusions are seen in around up t0 5% of NSCLC patients tumours and can be treated with targeted therapy (64, 65). *RET* fusion adenocarcinomas of the lung tend to have a solid, poorly differentiated microscopic phenotype and are more likely to occur in young never-smokers (66, 67). Other series show a correlation between *RET* fusion and a papillary phenotype with psammomatous calcification resembling papillary carcinomas arising in the thyroid gland (68, 69). Unlike in the setting of thyroid carcinoma, however, it is only *RET* gene fusions that are clinically actionable. *RET* fusion induced overexpression of RET protein can be detected by immunohistochemical methods but is not currently recommended by the ESMO as expression levels may be elevated without an underlying *RET* gene fusion lesion (49, 70, 71).

BRAF

Mutations in *BRAF* are identified in up to 5% of patients with NSCLC and often have a micropapillary architecture microscopically with a clinical history of smoking (72, 73). The use of immunohistochemistry for detection of the most common variant in *BRAF* (50%) seen in lung adenocarcinomas (V600E) is yet to be established in evidence (71, 74, 75). However, NICE recently approved the use of BRAFV600E IHC for screening melanomas for underlying *BRAF* mutations, recognising the clinical utility of rapid turnaround time and cost effectiveness compared to NGS, particularly for patients with a high burden of disease (76, 77). The indication for use of BRAFV600E IHC in the setting of NSCLC may change with additional evidence for impact for patients and may be more likely when multiplex immunohistochemistry methods become more routinely available in cellular pathology laboratories (6, 78, 79). Multiplexing IHC will enable use of a diverse range of antibodies, both diagnostic and predictive, to be used in small biopsy sample which are so common in the diagnostic setting of NSCLC (80).

MET

Mutations in splice sites within the *c-MET* proto-oncogene can lead to exon14 of the gene being skipped in transcription with the consequence of reduced degradation of the receptor (81, 82). *MET* gene amplifications have also been documented as a mode of acquired resistance to TKI therapy in NSCLC patients with somatic *EGFR* mutations (83). Immunohistochemistry for detection of MET fusion protein in NSCLC has only been used in a research setting to date. There is a lack of clinical evidence to support reliability of use in histopathology practice at the current time (71).

Additional Tier 1 Biomarkers not detectable by IHC

EGFR

The first somatic gene mutation to be targeted for therapeutic intervention in a setting of NSCLC was the epidermal growth factor receptor (*EGFR*) gene (84-88). *EGFR* somatic mutations are present in around 15% of the Caucasian population with NSCLC (89). The incidence rises in young, female never-smokers and can be as high as 60% in

Asian populations(90-92). Treatment with tyrosine kinase inhibitor drugs gives enhanced progression free survival (PRS) and overall survival advantages compared to standard chemotherapy in those patients with sensitising mutations (86-88, 93-97). Resistance to TKI therapy may develop during treatment, often but not limited to, development of a T790M somatic variant in *EGFR* (98-100). Development of second-generation agents, such as Afatinib, and third generation TKIs such as Osimertinib, have enabled patients to overcome this resistance by changing drugs (99, 101-104). Afatinib has a greater affinity for the intracellular tyrosine kinase domain of the EGFR protein and Osimertinib has irreversible binding (105). Initially patients were commenced on Osimertinib after developing resistance but use of Osimertinib was approved for first-line treatment during the coronavirus pandemic recognising the advantages of avoiding resistance (106-109). It should also be said that disease progression on TKI therapy can also occur through other mechanisms such as development of somatic *MET* amplification (110) or from histological transformation to small cell neuroendocrine carcinoma (100, 105, 111). Osimertinib has been approved by NICE for use as an adjunct to surgical resection in early-stage NSCLC. Combination somatic *EGFR* mutations, though uncommon, have been reported and may reflect sub-clonal populations within a single tumour and this may result in both difficulty interpreting the findings for clinical prediction and also variable responses to TKI therapy (112, 113).

Wild-type EGFR receptors are widespread amongst most epithelial types and organ systems (114). The EGFR protein is a transmembrane protein which binds to epidermal growth factors to cause cellular proliferation through phosphorylation and dimerization of the intracellular tyrosine kinase domain (114). There are two main types of clinically significant variants within *EGFR* gene, sensitising mutations which promote cellular proliferation and are considered drivers of carcinogenesis, and resistance mutations, for example T790M as described above which indicate a probable non-responsiveness to TKI therapies(115). Passenger mutations may also occur and be of no clinical relevance and germline mutations are not an indication for treatment in a setting of NSCLC (116). regarding interpretation of *EGFR* variants from next generation sequencing data. Clinically relevant mutations in *EGFR* occur in exons 18-21 which relate to the intracellular tyrosine kinase domain of the EGFR transmembrane protein (65, 117). The most common actionable sensitising mutations are small deletions in exon 19 and point mutations in exon 21 that result in amino acid change Leucine to Arginine at position 858 of the protein (L858R)(118). These mutations make up 85% of clinically actionable variants detected in somatic NSCLC tissue samples assessed in clinical trials of TKI therapies. The remaining 15% of mutations identified will include novel or rare variants and can be difficult to interpret regarding clinical actionability due to insufficient evidence in the medical literature (115, 119-121).

The method used to detect an *EGFR* mutation in NSCLC should be determined by the pathologist handing the specimen for diagnosis and requested in a reflex manner (122). Knowledge of the clinical urgency for reporting, particularly in advanced, stage 4 disease, and the amount of tissue available should play a key role in the decision-making process(123). The method of choice with be primarily between PCR, using primers of know actionable mutations, digital droplet PCR, pyrosequencing or NGS which will yield novel and rare mutations with precise information regarding the nucleotide change (124, 125). The alterations in the intracellular tyrosine kinase domain do not manifest in a protein alteration that can be reliably detected by immunohistochemistry and this is not clinically recommended (21, 65, 126).

KRAS

KRAS codes for a GTP-ase enzyme downstream in the signalling pathway from EGFR that influences cell proliferation and enhanced cell survival via the MEK/ERK cell signalling pathway (114, 127). Sotorasib was approved for use in the UK for patients with NSCLC with somatic G12C *KRAS* mutations early this year (128). The approval was based on evidence from the CodeBreaK100 phase 2 study of 126 patients that found a durable (12months) partial response in around a third of patients with somatic G12C *KRAS* mutation-positive NSCLC(129-131). Somatic *KRAS* mutations are more frequently encountered than *EGFR* and are said to be mutually exclusive (23, 65). This could be useful in designing a flow chart for single gene testing by rapid PCR whereby one refrains from testing *EGFR* and *BRAF* when a *KRAS* mutation is identified. It has been suggested by some authors that RNA sequencing should only be performed after exclusion of a DNA driver mutation in *EGFR*, *BRAF* and *KRAS* in the interests of cost effectivenss (23). It should be noted that patients are only eligible for Sotorosib therapy in the UK when they have relapsed following first-line immune checkpoint inhibitor and/or standard platinum-based chemotherapy treatments (128).

Conclusion

In conclusion, predictive biomarkers can be reported to oncologists with a short turnaround times when the assay is based upon immunohistochemical methods, for example ALK-1. This approach also has the advantage of being cost effective. The use of IHC can allow for full integration of relevant IHC findings to be given in one report with morphological and diagnostic IHC findings for ease of access and robust clinical governance. Some IHC assays are not recommended for routine use, such as EGFR, where next generation sequencing or PCR is a more reliable approach.

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