Clinically Actionable Biomarker Identification in Solid Tumour Diagnostic Cellular Pathology

Submission for Medical Doctorate by Published Works

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U.K.

2025

Word count: 15,651 excluding appendices and references.

References: 250

Content

Acknowledgements

Many thanks to Ms Sarah Saunders for her tireless enthusiasm and support in providing training for departmental staff in Swansea Bay University Health Board in using the Idylla™ instrument, console and explore software to conduct in-house, near patient PCR tumour testing. Thanks too to Sarah's colleagues, James Knowles, Lize Bollen and Helen Sumner for reviewing and accepting applications for investigator lead study resources in the form of testing cartridges which were provided free of charge.

Thanks to Ms Kate Hurlow and Mrs Kate Murphy for assistance with conducting Idylla™ platform tests. Thank you to all the laboratory staff, technical, scientific and medical, including management, who spent time listening to my arguments for in-house patient tumour testing. Across many disease types and biomarkers and in their many different and complementary forms.

Thanks also to Dr Claire Morgan for unwavering support and instruction in cancer genomics and agreeing to advise on the route to MD by publications.

And most grateful thanks to my long-suffering husband and children who have always supported my projects and given me the time to stretch my mind and academic interests without complaint. Without them, this work would not exist.

Summary of Works

The landscape of tumour testing for genomic alterations is developing at an exceptionally fast pace and applies to all solid tumours now that tumour agnostic testing is required in patients who have failed standard oncology treatments with evidence of clinical and radiological progression (1-5) .

The focus of the collection of the published works for presentation in this thesis examines how we might do things differently within histopathology laboratories to improve patient experiences, efficiency of workflows and clinical pathways for patients with non-squamous, non-small cell lung carcinoma (NSCLC) and metastatic malignant melanoma.

The following papers form the submission for a Medical Doctorate degree. The research and literature reviews investigate, evidence and improve biomarker identification commenced following discovery of rapid, near patient, fully automated technology at the Royal Brompton Hospital, Thoracic Pathology Update Course in London May 2016.

- Finall A., Jones, K. Applying bioethical principles for directing investment in precision medicine. *Clinical Ethics* 2020;**15**(1):23-28 (6).
- Finall A, Davies GJ, T Jones, Emlyn G, Huey P, Mullard A. Integration of rapid PCR testing as an adjunct to NGS in diagnostic pathology services within the UK: Evidence from a case series of non-squamous, non-small cell lung cancer (NSCLC) patients with follow-up. Journal of Clinical Pathology Jan 2022, DOI: 10.1136/jclinpath-2021-207987 (7).
- Finall A, Murphy K, Frazer RD. Improving care of melanoma patients through efficient, integrated cellular-molecular pathology workflows using tissue samples with low tumour nuclear content. Journal of Clinical Pathology Apr 2022, DOI: 10.1136/jclinpath-2022-208194 (8).
- Bennett P, Finall A, Mederios F. Gerrard G, Taniere P. Inadequacy of PCR genotyping in advanced non-small cell lung cancer: EGFR L747_A755delinsSS exon 19 deletion is not detected by the real-time PCR Idylla™ EGFR mutation test but is detected by ctDNA next generation sequencing and responds to osimertinib. Sub-title: The apparent "Inadequacy of PCR genotyping in advanced non-small cell lung cancer": A counter perspective. European Journal of Cancer (9).

- Finall, A. RNA next generation sequencing in the somatic molecular testing of non- small cell lung cancer (NSCLC): Is it time to re-consider testing options for improved patient care? Journal of Molecular Pathology, 2022, 3,307-318. DOI: 10.3390/jmp30406 (10).
- Finall A, Hurlow, K, Leopold G, Elazzabi T, Goldsmith IR, Basu S. Analysis of EGFR mutation by rapid PCR methods yield better quality results using intra-operative frozen section tissue in early stage, non-small cell lung cancer patients. IJCMCR, 2023;25(4):0. DOI: 10.46998/IJCMCR (11).

The polymerase chain reaction (PCR) has been used for many years to determine clonality in in the clinical diagnosis of lymphoma, particularly low-grade lymphoma (12-15). Initially, polymerase chain reaction (PCR) to support histopathological diagnosis required fresh tissue for success but over time the technique has been modified for use with formalin fixed, paraffin embedded (FFPE) tissue samples (15, 16). The repertoire of genomic tests required in histopathology has expanded exponentially and includes multiple potential methods and indications. Examples include interphase fluorescence in-situ hybridisation techniques (FISH) for diagnosis and sub-typing of sarcomas (17, 18) and large panel DNA next generation sequencing (NGS) in lung cancer to highlight subsets of patients with particular single nucleotide variants in gene hotspots that confer likelihood of response to particular drugs as part of precision medicine (1, 19). This research and service development projects published, focus on precision medicine, or therapeutic, aspects of somatic tumour analysis. They examine patient and tissue factors that inform the decision-making process to select the right test, at the right time, for the right patient (20, 21).

Somatic gene testing in lung cancer

Lung cancer has the highest mortality compared than any other malignancy type in the UK (22). Survival outcomes from non-squamous, non-small cell lung carcinoma (NSCLC) in the UK are also amongst the poorest of developed nations across the world, including Europe (23, 24). The reasons for this difference in outcome lie largely in detection at late stage as early-stage lung cancer is often asymptomatic (23).

In Wales, 49% of patients present with stage 4 NSCLC and this may be as high as 56% in areas of socioeconomic deprivation where smoking rates are higher (25). Work is ongoing to develop a lung health check based on low-dose computed tomography (CT) for earlier detection and potentially curative surgery. There is a need to focus on expediting the diagnostic pathway from primary care referral onwards to realise benefits of this form of "targeted health check" which is often referred to as a form of screening (26-28). Key 'bottle necks' in the diagnostic pathway are insufficient capacity in radiology and cellular pathology plus delays in reporting molecular pathology (29). This situation was compounded by backlogs created during the COVID-19 pandemic (30, 31).

The requirements for molecular pathology in a setting of NSCLC have expanded rapidly over the past 10 years (1). The European Society for Medical Oncology (ESMO) ranks genomic variants in somatic genes of tumours according to clinical relevance and actionability drug treatment (32). Tier one variants have a matched drug therapy and have been shown to benefit patients in randomised clinical trials (24). Tier 2 somatic genomic changes are yet to be shown to have a clinical benefit when the matched drug is given to patients and Tier 3 variants are thought likely to show efficacy in trials but in patients with other tumour types outside the focus of interest. Tier 4 variants are being analysed in pre-clinical studies for drug development and Tier 5 variants have a drug match but show no objective response in patients in clinical trial data (33).

The current tier 1 somatic gene testing requirements for all patient malignancies are published on the NHS England website, 'National Genomic Test Directory' and are regularly updated (34). At the time of writing, tier 1 requirements in non-squamous NSCLC include testing for actionable somatic mutations in epidermal growth factor receptor (*EGFR*), B-raf proto-oncogene (*BRAF*) and K-ras proto-oncogene (*KRAS*) in

addition to structural variants in anaplastic lymphoma kinase (*ALK1*) ROS proto-oncogene 1 (*ROS-1*), Met proto-oncogene receptor tyrosine kinase (*MET*) exon 14 skipping lesions, Ret proto-oncogene (*RET*) fusions and fusions in neurotrophic receptor kinase (*NTRK*) gene fusions (35-41). This range of gene analysis also may be applied to patients with squamous carcinomas but usually only if they have a clinically high likelihood of having a targetable mutation, for example if the patient is younger than 50 years or is a non-smoker (34). The NHS test directory also lists NTRK testing in patients with small cell neuroendocrine carcinoma in whom all previous lines of therapy have failed (34, 42-44). Retinoblastoma gene (Rb) sequencing may also be useful for resolving diagnostic difficulty at the morphological level (34, 45). Tier 1 evidence is required for the Medicines and Healthcare products Regulatory Agency (MHRA) to approve oncological drugs for therapeutic use in patients in the UK and also forms the basis of recommendations and guidelines set out by the National Institute for Health and Care Excellence (NICE)(46-49).

The key performance indicator is for results from these tests being available to the lung cancer MDT within 10 working days in Wales (29), as set out in the National Optimal Pathway (see figures 1 and 2). There are a number of issues not addressed by the current pathway that need closer scrutiny; are any of our patients adversely affected by a turnaround time of 10 working days, are we able to meet the tissue requirements for two NGS panels and could we do things differently and better?

Motivation for the study series

Anecdotally, it was noted in lung cancer MDT that some patients were deteriorating rapidly with NSCLC, and some were deceased before the NGS reports were available from the centralised sequencing laboratory. An illustrative case in point was a middle-aged teacher, still actively working, who presented with stage 4 disease when a histopathological diagnosis of primary pulmonary adenocarcinoma of lung was made. He had a good performance status, PS0, despite brain metastases but died within two weeks of a request for *EGFR* somatic testing. This patient's tissue sample had an exon 19 deletion in EGFR and could have been treated with Osimertinib, a tyrosine kinase inhibitor which crosses the blood-brain barrier. Osimertinib is licensed for first line treatment of NSCLC which harbors an actionable somatic mutation in the EGFR gene (47). Up to 70% of patients with an actionable mutation in EGFR respond very well to TKI therapy with progression free survival (PRS) and overall survival advantages and milder side effects (50). This case was part of the motivation for looking at more rapid somatic mutational testing in the histopathology laboratory.

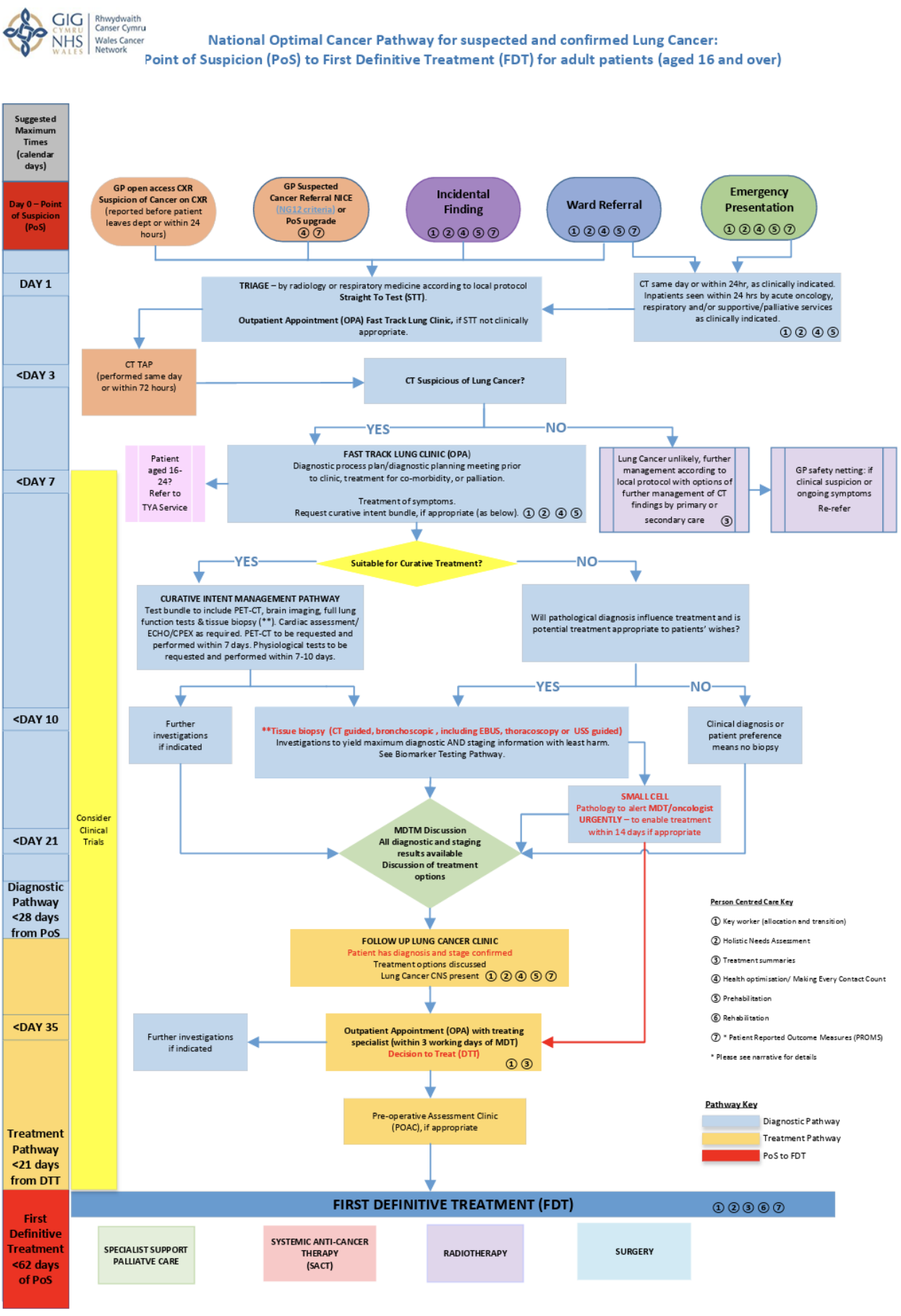


Figure 1: National Optimal Pathway for Lung Cancer. Wales Cancer Network 2022 (51).

Options Appraisal

An options appraisal was conducted in Swansea Bay University Health Board to consider in-house, rapid PCR testing. We examined feasibility of the Roche Cobas assay (38-41), Qiagen's Therascreen assay and the Biocartis Idylla *EGFR* test. At that time in 2018, *EGFR* was the only gene variant treatable with novel tyrosine kinase drugs so focussed on assays assessing this gene only. Cobas and Therascreen tests require batching of samples and DNA extraction in a molecular grade laboratory with a turnaround time of approximately 6 hours in both cases. Idylla, in contrast, allowed for individual testing on a fully automated system whereby DNA extraction is conducted within a closed cassette environment. The turnaround time for reporting from Idylla for EGFR mutation is 2 hours. This method was deemed most feasible for the Health Board given constraints around suitable sites for DNA/RNA extraction and staffing shortages. There was no requirement for batching samples or performing nucleotide extraction using the Idylla platform.

Being able to present single gene testing results to an oncologist on the same day of request represented an opportunity for patients to access appropriate treatment in a timely fashion. There is evidence that some patients start chemotherapy to bridge the waiting gap for somatic gene testing results to become available (52). Real-world practice evidence examining patient outcomes shows that any survival advantage from tyrosine kinase inhibitor (TKI) therapy is lost if a patient is switched to TKI therapy from chemotherapy whilst waiting for their somatic mutational analysis to be performed (52). The NHS in England acknowledges that some patients may be too ill to wait for next generation sequencing (NGS) results and have proposed a 'salvage pathway' for rapid single gene testing where appropriate (53).

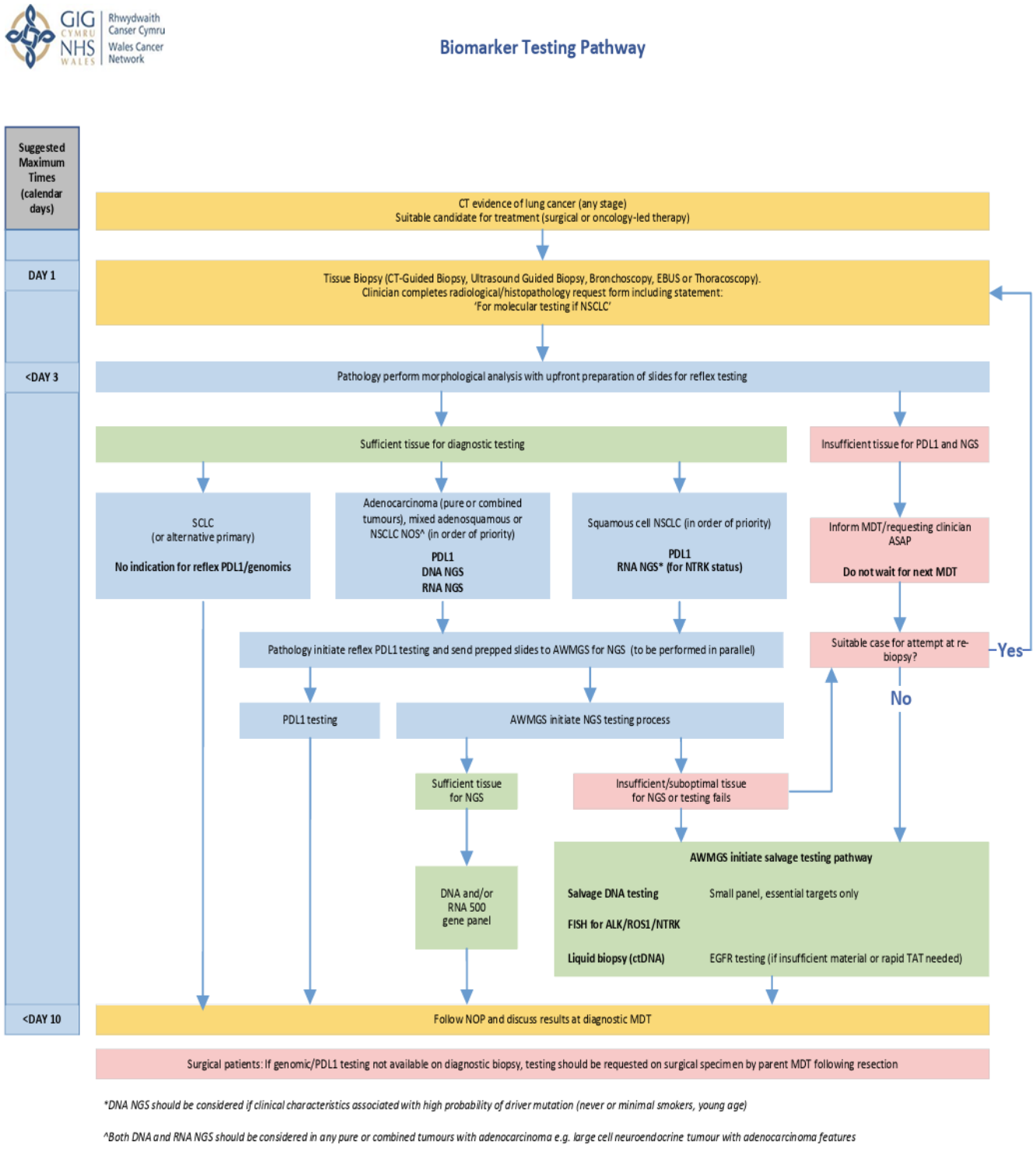


Figure 2: Biomarker testing pathway. Wales Cancer Network 2022 (51).

Malignant melanoma

Malignant melanoma is an aggressive non-epithelial malignancy that most commonly arises from the skin but can also occur on mucosal surfaces, conjunctiva and in subungual regions (54). There are four main histopathological patterns of disease arising in the skin, superficial spreading malignant melanoma, lentiginous, nodular and acral melanoma (55, 56). Patients presenting with local and distant metastases have two oncological treatment options; immune checkpoint inhibitors or Dabrafenib (+/- in combination with a mitogen activated protein kinase (MAPK/MEK) inhibitor) which targets melanomas with specific single nucleotide mutations in the *BRAF* gene (49, 57-61). The most common mutation occurring in *BRAF* in melanoma leads to V600E, a substitution of valine for glutamic acid at the 600 amino acid position of the BRAF protein (62). The V600E mutation leads to constitutive intracellular signaling in the MAPK/ERK pathway promoting cell proliferation and division (63). Blocking this signaling leads to a slowing or halting of metastatic melanoma progression and enhanced overall survival in 40-50% of eligible patients (63, 64). Eligibility is defined as the presence of a V600 mutation and is detected in around 50% of somatic melanoma tissue samples (49).

Using the fully automated PCR platform Idylla™ to test for *BRAF* mutations in melanoma has been shown to significantly reduce the time to initiate Dabrafenib therapy as compared with both immunohistochemistry (IHC) and NGS analysis of *BRAF* (65). The authors indicate that further studies are required to validate the assumption that patient quality of life, diagnostic experience and survival outcomes are positively affected by reduced time to starting treatment as their study was small and the patient groups not clinically comparable. Investigators in Europe have explored the integration of the Idylla *BRAF* assay alongside NGS for those patients with insufficiently good quality DNA requirements for the NGS in a setting of colorectal adenocarcinoma (21, 66-68). They were able to salvage 73% of the tissue specimens that were inadequate for NGS. In the combined NRAS/KRAS/BRAF cartridge designed for colorectal carcinoma, however, the tissue tumour nuclear content (TNC) requirements are as low as 10% (69). In a setting of malignant melanoma, the minimum TNC requirement is 50% according to the manufacturer's instructions for use (70). For the Idylla™ *BRAF* assay to be useful to patients and clinicians treating melanoma patients, we assessed whether valid results were achievable using formalin fixed paraffin embedded (FFPE) melanoma tissue samples with less than 50% TNC and showed that they were (8).

Clinical Guidance in Lung Cancer Diagnosis and Management

The suspected cancer pathway produced by the NHS Wales Health Collaborative is a guidance document with timeline targets for steps in the patient care pathway to ensure quality and safety of patients. The document was adopted by all health boards in Wales with respect to all cancer patients. Individual cancer types have National Optimal pathways to reflect the detailed investigations for site specific cancer patients. Welsh Government collect data for comparison against these waiting time targets and publish the data online (51). Example figures for July and August 2022 show that 51% of lung cancer patients with suspected lung cancer started first treatment within 62 days of first being suspected of having a malignancy (51). This compares with 53.5% for all tumour sites for the same period. At first sight this figure seems appalling, but 323 patients were investigated and told they did not have lung cancer (51). Welsh Government figures do not give an indication of what percentage of the total number of referred patients this reflects. It does give an indication of the level of investigation that is carried out on an urgent, suspected cancer basis within the system that can be reassured. The move from cancer waiting times to the single cancer pathway measuring from *point of suspicion* of malignancy was intended to more accurately reflect the patient experience and the workload of diagnostic health services such as radiology and cellular pathology. Recent data have exposed pressures on the diagnostic pathway as a consequence of backlogs generated during the coronavirus pandemic, see figure 3 (51).

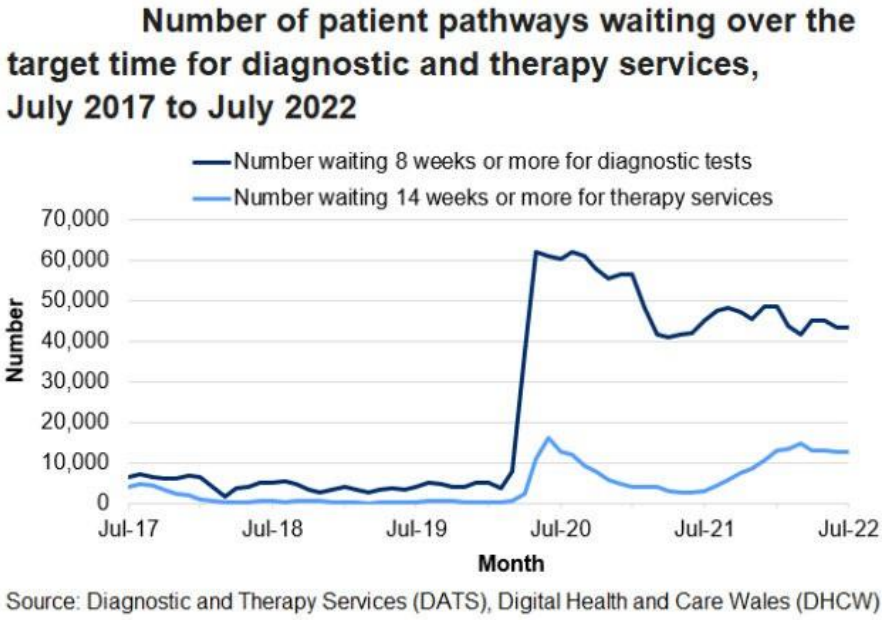


Figure 3: Data from NHS Wales activity and performance summary during the COVID19 pandemic showing impact on waiting times for diagnosis (dark blue line) and treatment (light blue line). Source: National Optimal Pathway for Lung Cancer, Welsh Government (51).

There is no current National Optimal Pathway for melanoma or other skin malignancies at the present time. The National Optimal Pathway (NOP) for lung cancer advises that patients be investigated with a tissue biopsy for diagnosis by day 10 from the point of suspicion of malignancy if the patient agrees and is well enough to proceed with the intention of radical treatment (51). All diagnostic and therapeutic information from tissue biopsies needs to be available by day 21 for discussion of treatment options at MDT (51). See figure 2. Diagnostic biopsy samples include computer tomographic (CT) guided lung core biopsies, endobronchial biopsy and fine needle aspiration cytology. All these samples may be very small and may or may not contain malignancy either at all or in sufficient quantities to support additional biomarker testing (51, 71, 72). See figure 3. The NOP biomarker pathway acknowledges the importance of cutting slides up-front for timeliness in sending tissue away to an external laboratory for molecular studies and that an assessment of quantity of tissue and pathologists initiated reflex requesting are also important to achieve targets of the NOP (72). See section on tissue processing for further details.

The biomarker pathway outlines a requesting pathway subdivided by histological subtype of malignancy. If the malignancy is small cell neuroendocrine carcinoma, then NTRK may be the only relevant biomarker for testing, as in all other solid malignant tumours, when all other standards treatments have failed (43, 44) It is possible to use pan-TRK

immunohistochemistry as a rapid screening tool to identify NTRK fusion negative cases (44, 73-76). A morphological diagnosis of small cell neuroendocrine carcinoma on H&E section must be rendered quickly (77). There is a national standard of starting treatment with standard intravenous chemotherapy plus etopiside within 2 weeks of diagnosis for best patient outcomes (77, 78).

Programmed death ligand 1 (PD-L1), is an immunohistochemical stain and reported alongside the morphological diagnosis in the pathology report (79-81). The process takes 1-2 days and consumes a single 3-4µm section of FFPE tissue. See figure 4. This is required in all non-small cell lung carcinoma (NSCLC) including squamous histotype (80). Squamous carcinomas arising in young, female never or light smokers may also require a full DNA and RNA panel of predictive biomarkers similar to the requirements for non-squamous NSCLC (34). Identifying effective, novel therapeutic targets in squamous carcinoma of the lung is an urgent clinical need (82). Potential candidate biomarkers from phase one clinical trials include cyclin dependent kinases (*CDK*) 4 and 6, and fibroblast growth factor receptor (*FGFR*) status in addition to *TP53, CDKN2A, PTEN* and *PIK3CA* (82, 83). Much of the therapeutic evidence of treating squamous carcinoma of lung with TKIs comes from case reports and small series (84-88). Most clinicians would agree that the best medical evidence for treating patients with squamous carcinoma comes from well powered clinical trials (89). Many oncologists would also agree, however, that is not unjustified, on a case-by-case basis, to consider off license use of sequencing data to provide salvage therapy on a compassionate basis (90-93). Some report that as much as 67% of oncology drugs were prescribed off-label and 15% were prescribed on a compassionate basis (93).

Oncologists must be mindful of the potential implications of somatic tumour genome profiling for understanding the patients' germline constitution. For example, identification of a PTEN mutation in a lung squamous carcinoma would reflect a germline alteration in 10% of patients (94). Some patients will need to be counselled regarding familial implications following somatic tumour sequencing. Some authors argue that patients may be more appropriately counselled prior to testing as they have a 'right not to know'(95, 96). Pre-test genetic counselling for all oncology patients could potentially overburden budget-restricted healthcare systems however (6). The NHS test directory for solid tumour testing advocates the identification of the same gene variants and fusions as for non-squamous, non-small cell carcinoma in those patients with a non-smoking history under the age of 50 (34).

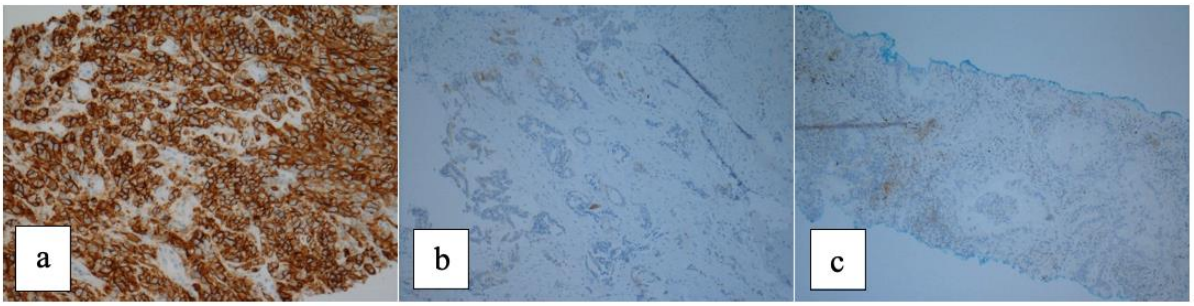


Figure 4: *PD-L1 immunohistochemistry in NSCLC showing total percentage scores (TPS) of 4a) 100%, high expressors, 4b)5 % low expressors, and 4c) <1%, negative expression at the lowest reporting threshold. Care needs to be taken in low expression and negative cases that background staining in inflammatory cells, as seen in c), is not counted erroneously in the PD-L1 TPS score. Background inflammatory cell staining can be a useful internal control in negative cases (4c).*

For non-squamous NSCLC patients, the recently published lung biomarker pathway indicates that one should proceed straight to DNA and RNA NGS panel studies to detect relevant genomic somatic biomarkers to aid treatment decisions immediately after PD-L1. There is no acknowledgement in the Welsh National Optimal Pathway of widespread use of immunohistochemistry to assess ALK-1, NTRK, RET and ROS-1 fusion status across the UK in recognition of cost savings and time efficiency for patients (51).

The progress of a tissue biopsy sample through a cellular and molecular pathology laboratory system is complex and affects patient outcomes and frontline clinical experiences (8). The current system is for a centralised molecular laboratory in Cardiff with cellular pathology laboratories from Wales submitting samples to this centralised 'hub' for molecular testing. This model reflects the GLH model of testing in NHS England. The service in Wales is commissioned by the Welsh Heath Specialised Services Committee (WHSSC) who receive funding from all the Health Boards in Wales to provide molecular testing services for their patients. This model does not account for funding required to prepare slides in cellular pathology laboratories to send to the centralised molecular hub nor does it cover specialised companion diagnostic immunohistochemistry such as PD-L1 and ALK-1.

Pre-analytical phase of cellular pathology

Decision to biopsy a patient with suspected lung cancer occurs at MDT in a context of imaging findings and an understanding of the clinical context of the patient. Only patients who want to proceed with radical treatment options will go on to biopsy. Clinically, patients also need to be well enough for oncological drugs and/or surgical intervention depending upon stage and performance status.

Diagnostic biopsy samples are taken by an interventional radiologist for peripheral lesions (CT or USS guided core biopsy from metastatic site or lung primary) or respiratory physician for central thoracic lesions via endobronchoscope (EBUS or bronchial biopsy). These small specimens are placed in 10% neutral buffered formalin for fixation between 6 and 72 hours (97). Processing to an FFPE tissue block requires automated dehydration in alcohol alternating with removal of lipids using solvents such as xylene. Standard processing occurs on an overnight cycle but short cycle processing is also processing (3 hours) for small well- fixed specimens (98). Embedding fully processed samples into paraffin wax is the final step before sections can be cut at 3-4µm thick on a microtome. Pre-cutting of slides before requests for molecular studies are preferred to prevent waste of FFPE tissue in small biopsy samples through trimming alignment at the microtome (99, 100). Laboratory handling of cytology specimens is a different pre-analytical process. In a context of NSCLC, this is commonly a pleural fluid sample. These samples may be important for patients where a tissue biopsy diagnosis has failed or in cases of stage 4 disease with no intention for radical treatment. Cells are drawn through a fine filter in a Thinprep^{TM} processor and deposited onto a slide for Papanicalou staining. Papanicalou stains give good nuclear chromatin detail for assessment by a pathologist. The remaining serous fluid is spun down and supernatant discarded. A sample of the cells in the centrifuged pellet are directly spread onto another slide, dried and stained with Giemsa, a cytology stain that gives good architectural features. If after first assessment the cellular features are suspicious for malignancy, the cell pellet may be combined with bovine prothrombin and fibrin and then processed into a cell block using the protocol as described above for tissue specimens. This allows sections to be cut for immunohistochemistry for diagnosis and theranostics if appropriate. Caution must be taken in interpreting immunohistochemistry from cell blocks as the deeper section appearances change from section to section depending upon the cellularity of the specimen. Furthermore, not using standard formalin fixation protocols in generating cell blocks can resulting in over retrieval of antigens using standards heat methods (101, 102). False positive IHC findings can occur using cell

blocks and this has been reported in a context of ALK-1 IHC (103). Some advocate use of direct cytological smears for use in ALK-1 detection by FISH or NGS rather than cellblocks for this reason (104, 105).

The analytic phase of tissue diagnostic involves subjective human assessment of glass microscope slides. In some centres, primary assessment using digital images is commonplace (106). To reach a diagnosis, a cellular pathologist forms a differential diagnosis based on clinical information on the request form followed by assessment of the macroscopic appearances in combination with the microscopic appearances and additional information from IHC, FISH, electron microscopy (EM), PCR, NGS, special histochemical stains (for example, Perls Prussion blue for iron) and/or flow cytometry. Diagnostic IHC is limited to TTF-1 and p40 for determination of sub-type of NSCLC where morphological features indicate an epithelial malignancy. A wide panel of immunohistochemistry may be required for a diagnosis of metastatic melanoma (107). Where an in-situ epidermal lesion is identified next to an invasive lesion in primary malignant melanoma, a smaller panel of IHC including MelanA, cytokeratins and S100 may suffice as the presence of an in-situ lesion is a strong diagnostic clue which also indicates site of origin.

Post -analytical phase of cellular pathology

A report is constructed detailing all information from tests carried out on the tissue or cytology sample. Integrating all information relating to a single tissue specimen is recommended by the Royal College of Pathologists in the UK (108). Standalone publication of cancer genomic information is not recommended as the relevance of the findings cannot be accurately interpreted without reference to the diagnosis and may cause clinical confusion. Furthermore, it is important that the genomic information reports related to the correct specimen number to ensure clinical governance standards are met. Cellular pathology reports have a unique identifying number which corresponds to that on the request form and specimen pots. The final report is published for viewing by healthcare colleagues on the hospital laboratory information system (LIMS). The reports and specimen microscopic slides are subsequently reviewed by a sub-specialty specific pathologist for discussion at MDT where nursing staff, surgeons, physicians, oncologists and radiologists develop the best management plan for each patient based on case review and discussion (109).

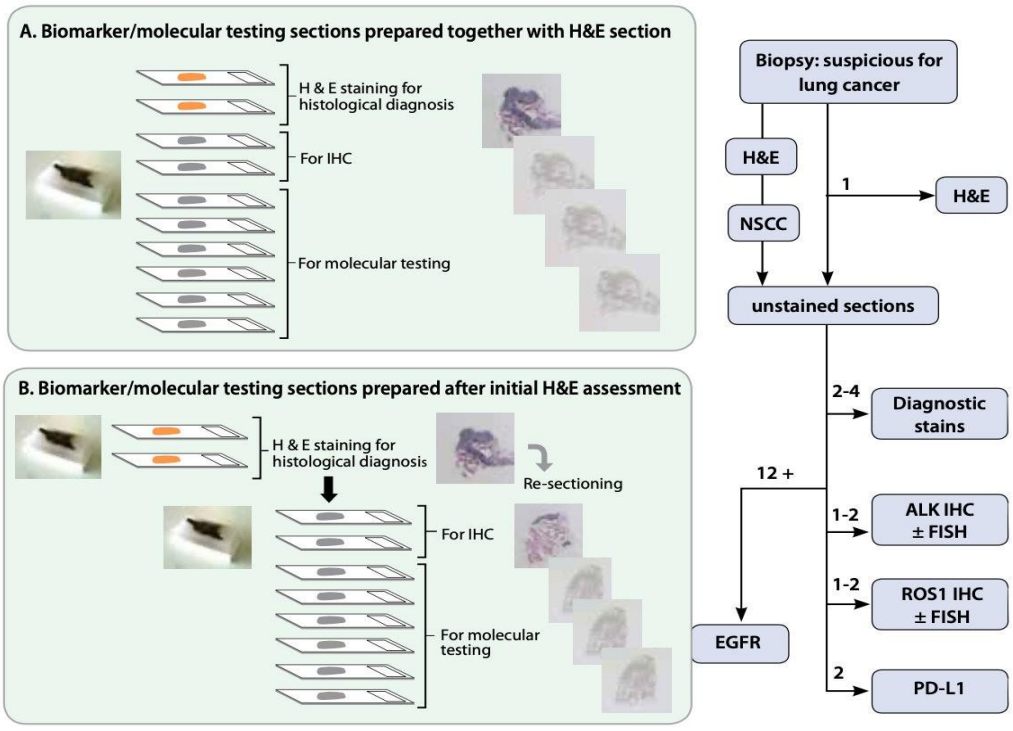


Figure 5: Diagrammatic explanation of up-front cutting of formalin-fixed paraffin-embedded (FFPE) tissue blocks to prevent wasting diagnostic tissue. Source: IASLC Atlas of EGFR testing in lung cancer (110).

Molecular testing methods and reporting

The current system for reporting solid tumour genomics varies between laboratories across the UK. In general, many cellular pathology laboratories received standalone genomic reports which have been issued direct to the patients oncologist before integration into the tissue report. RCPath guidance on solid tumour genomic reporting indicates that integration of morphology and molecular findings must take place for each tissue specimen to avoid misinterpretation in a climate of multiple biopsies for each patient with a malignancy and in an era where some patients have survived more than one cancer (108).

Furthermore, there have been instances of molecular markers being requested for *diagnostic* purposes rather than therapeutic purposes which have led to incorrect information being published in gene analysis reports conducted in a centralized laboratory. An example includes a case of pleural biopsy with suspected sarcomatoid mesothelioma being tested for *SS18* gene fusion to exclude a differential diagnosis of synovial sarcoma. The genomics report in this case stated “Indication: Sarcoma” as a report heading with consequential confusion for the patient and their healthcare professionals. Some cellular pathology diagnoses are made by excluding morphologically similar entities with a known specific molecular or immunohistochemical phenotype.

Chapter 2
Publications in Chronological Order

i) *Finall A, Jones K. Applying bioethical principles for directing investment in precision medicine. Clinical Ethics 2020;15(1):23-28.*

This paper discussed ethical issues surrounding genomic testing in a wide variety of cancer settings with a focus upon turnaround time of reporting. The need to balance the needs of many (utilitarianism) against a few (egoism) is the ethical principle that primarily underpins the discussion around somatic genomic testing, its timeliness and economics, particularly with respect to functioning of the NHS in the UK.

The paper focuses on the ethical considerations around detection of solid tumour biomarkers, mainly nucleotide-based, in malignant tissue samples that can give prognostic and/ or therapeutic information rather than markers that may be required for making a pathological diagnosis. The number of predictive biomarkers required to inform therapeutic management of patients with malignancy has expanded significantly over the last 30 years (111, 112). Many of the biomarkers that are actionable (meaning can be used to determine eligibility for novel oncology drugs; so-called predictive biomarkers) are rare and the chemotherapeutic drugs new and very expensive. This situation leads to difficult strategic decisions around developing precision medicine services. Do we invest in expensive tests like next generation sequencing to find occasional mutations in common malignancies that could be controlled (reduce disease burden) from very expensive drugs that extend life by a couple of years? On a background of insufficient healthcare staff and NHS services increasingly struggling to cope with the health demands of an aging population what areas of clinical practice can we focus on for maximum benefit across the NHS? (113-117)

The manuscript was written with Kerina Jones, Professor of Population Data Science at Swansea University, and is a discursive piece discussing core principles of medical ethics, promoting beneficence, non-maleficence, justice and autonomy in the setting of NHS-based medical practice in the UK (118-120).

We discuss how decisions around testing solid tumour samples for genomic biomarkers are often taken without the participation of patients who are thereby denied a degree of autonomy in their care (109, 121). It might seem that direct to consumer testing (DTC) offers a beneficial counter stance to reflex requesting by pathologists but there is evidence to suggest that patients can be misled by DTC marketing information and/or a lack of information (122, 123). Furthermore, some argue that being in control of choices around genomic testing are a form of procedural justice, from a medical ethics point of view. And, whilst wanting to test patient tumour samples to detect potential targets for beneficial treatment represents healthcare professional wanting to enact beneficence and nonmaleficence, it does so at a significant financial cost to the NHS (124).

One of the products of this manuscript was to identify a key research question that had limited evidence in the medical literature. The ToGA study was a phase 3 clinical trial that examined potential benefit of Trastuzumab in combination with chemotherapy vs chemotherapy alone for the treatment of HER-2 positive gastric and gastro-oesophageal junction adenocarcinomas (125). Rather than genomic biomarkers being a key to highlighting patients for treatment, an immunohistochemical (IHC) assay reflecting gene amplification and overexpression of HER2 protein on the membrane of malignant cells was scored by pathologists. Overexpression of the protein assessed by microscopic evaluation correlates with gene amplification at the somatic ErbB2 gene at the DNA level (126). The clinical trial reported that the time to receive the report for HER2 status of the tumour samples took two weeks and during that time a quarter of patients clinically deteriorated and became ineligible for treatment (125). Patients need to be physiologically well enough to have a reasonable likelihood of successful oncological treatment and withstand potential side effects. This is assessed by Eastern Co-operative Oncology Group (ECOG) performance score (PS) (127) and patients in the clinical trial had to have a PS score 0 or 1 to qualify for inclusion. A performance score of 0 reflects no limitations in activities of daily living. PS1 equates to a restriction in strenuous physical activity only but with retained capacity to perform light work. PS2 means a patient is unable to work but able to look after their self-care needs independently whilst a PS3 score reflects a loss of ability to self-care. Patients in the PS3 group are usually con fined to bed or chair for less than half their waking hours. PS4 patients are confined to bed or chair for more than 50% of their waking hours in contrast and PS5 is sometimes, but this is rarely used in place of the term deceased. There was a gap in the medical literature. It was not known what proportion of patients with

lung cancer deteriorate rapidly and did a proportion of our patients miss out on tyrosine kinase inhibitor therapy during the wait for NGS panel reporting of EGFR. This led to our publication entitled, “Integration of rapid PCR testing as an adjunct to NGS in diagnostic pathology services within the UK: Evidence from a case series of non-squamous, non-small cell lung cancer (NSCLC) patients with follow-up” (7).

ii) Finall A, Davies GJ, T Jones, Emlyn G, Huey P, Mullard A. Integration of rapid PCR testing as an adjunct to NGS in diagnostic pathology services within the UK: Evidence from a case series of non-squamous, non-small cell lung cancer (NSCLC) patients with follow-up. Journal of Clinical Pathology Jan 2022, DOI: 10.1136/jclinpath-2021-207987.

The purpose of this work was to answer the question, 'Do patients deteriorate and miss out on therapeutic opportunities whilst waiting for next generation sequencing (NGS) to be reported?'. We sought to quantify the number of patients this may apply to, if relevant, and consider if there were ways to predict which patients were most likely to deteriorate clinically. The initial assessment was to look at turnaround times for reporting NGS and examine the proportion of patients who deteriorated according to clinical records of performance status. The key performance indicator for reporting NGS by central genomic hubs, distinct and separate entities to cellular pathology laboratories, is 10 working days according to Welsh standards (29, 128). A recent report published by the UKLCC further makes the point that turnaround times within 14 days are required for better patient outcomes (129). The recent Darzi report on the state of the NHS in England indicates that just 60% of eligible patients access genomic panel testing in a setting of lung cancer (130).

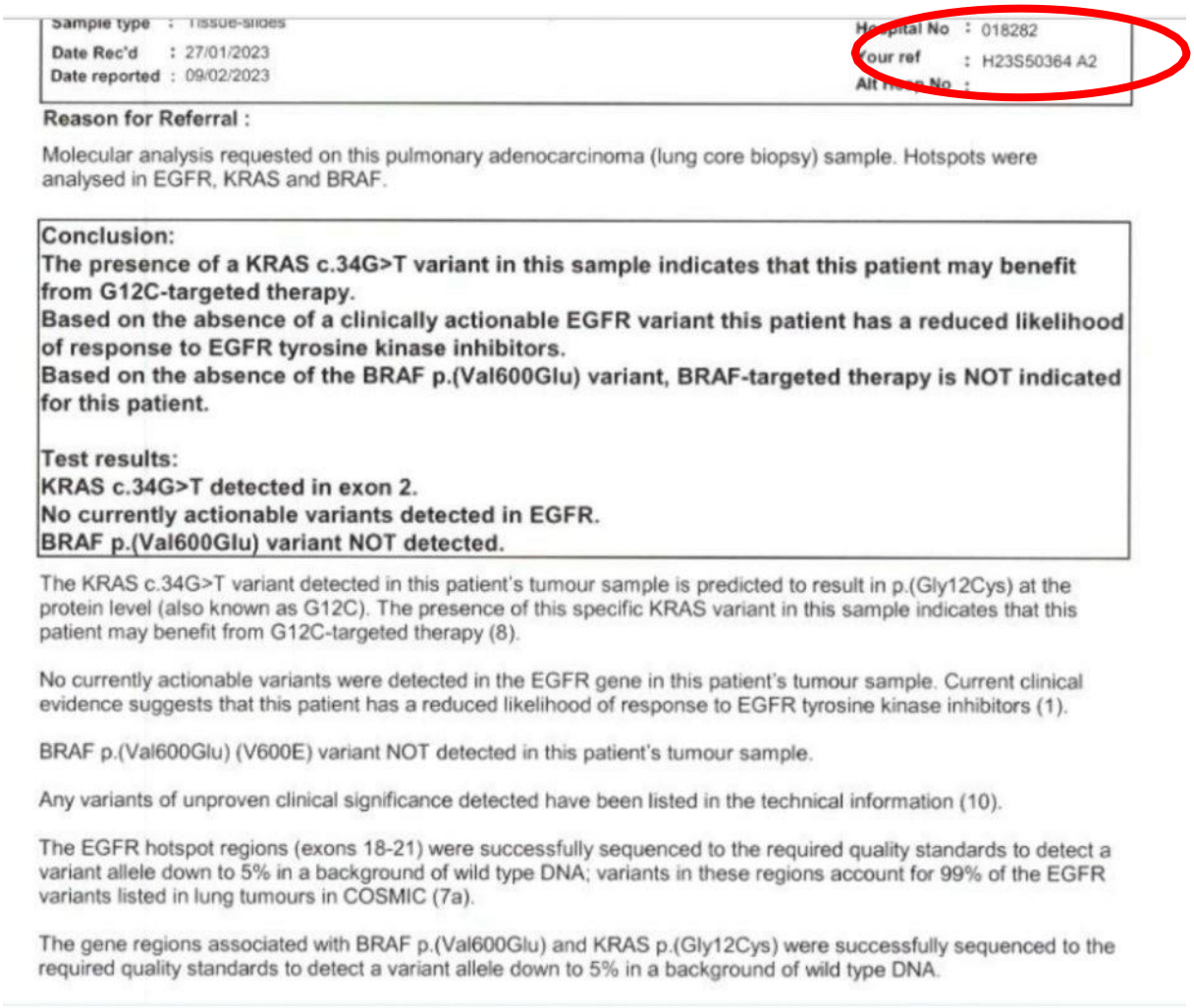
This paper examines turnaround time for gene panel testing in patients receiving a pathological diagnosis of lung cancer in Wales. When to start calculating time to report is an important consideration in data collection. Genomic hibs may only consider tuime to report from receipt of tissue scrolls or slides at the genomic laboratory hub site reception. The starting time point may not include time taken for cellular pathology laboratories to prepare slides nor does include transport time, both of which can be considerable (129). Requests for NGS are made by the diagnostic pathologist reporting the case at the time of diagnosis by an in-house electronic request form for additional laboratory work to be carried out on a "reflex" basis. "Reflex" requesting in the medical literature refers to immediate requesting of ancillary investigations before MDT discussion to save time. With clinical information on histopathology request forms often being limited, this frequently means requesting by pathologists is blind to the stage of malignancy and performance status of the patient (131).

Our paper illustrates the real-life turnaround time for reporting NGS in our area was 17 calendar days. We chose calendar days as a more realistic reflection of the ongoing proliferative capability of lung cancers during the weekend days to make our work patient-

centred. We set the starting point for turnaround time calculation for NGS reporting the time from which the samples were sent from our laboratory. If one includes the time taken by the biomedical scientists to cut the tissue sections and send the tissue to another city, the mean turnaround time for NGS reporting was 23 days. It should be noted that the genomic hub producing reports in this study gives a standalone report for somatic gene analysis directly to the oncologist. The turnaround time calculation does not include travel time for a paper report by standard mail delivery. There are potential governance issues with release of standalone genomic reporting of somatic tumours as pathologists loose the opportunity to sense check mutually exclusive gene driver events and confusion may also arise regarding which specimen has been tested in a context of multiple tests for the same disease and different tests in synchronous malignancies (132). The only reference to the tissue histopathology report on an external laboratory standalone gene report is the FFPE block number as illustrate in figure 6 below in the red ellipse.

We found that 18% of our patients suffered a rapid clinical deterioration as defined by a fall in two performance points within an 8-week period. Three quarters of patients in this group had a diagnosis of stage 4 radiological lung cancer at presentation and 33% of this group were deceased before the NGS report was available and 16% (three patients) had an actionable EGFR mutation detected by NGS. Eleven percent of patients in the deterioration group had actionable mutations but had become ineligible for treatment by the time the report was available and died shortly afterwards. The genomic report turnaround time for the 3 patients with actionable variants in EGFR was 15, 18 and 28 calendar days respectively. It should be noted that patients with poor performance status and an actionable EGFR mutation (defined as ESOG score 3 or more) have an overall survival of between 12.7-18.3 months with TKI therapy compared with 1.4-3.6 months in those poor PS patients who were not treated giving an evidence basis to support treatment of patient with a PS worse than or equal to 3 (133).

A comparative analysis of several clinical parameters across the deterioration group and the non-deterioration group highlighted that radiological stage of lung cancer at diagnosis and first MDT discussion was a potential predictor of rapid clinical decline and that stage 4 presentation was a risk factor for rapid decline ($p<0.005$). Radiological stage at present reflects systemic burden of disease with, for example, stage 1 patients having primary lung cancers less than 3cm in size with no metastases and stage four patients having multiple distant metastases.



Sample type : Tissue-slides
Date Rec'd : 27/01/2023
Date reported : 09/02/2023
Hospital No : 018282
Your ref : H23S50364 A2
Alt Hosp No :

Reason for Referral :

Molecular analysis requested on this pulmonary adenocarcinoma (lung core biopsy) sample. Hotspots were analysed in EGFR, KRAS and BRAF.

Conclusion:
The presence of a KRAS c.34G>T variant in this sample indicates that this patient may benefit from G12C-targeted therapy.
Based on the absence of a clinically actionable EGFR variant this patient has a reduced likelihood of response to EGFR tyrosine kinase inhibitors.
Based on the absence of the BRAF p.(Val600Glu) variant, BRAF-targeted therapy is NOT indicated for this patient.

Test results:
KRAS c.34G>T detected in exon 2.
No currently actionable variants detected in EGFR.
BRAF p.(Val600Glu) variant NOT detected.

The KRAS c.34G>T variant detected in this patient's tumour sample is predicted to result in p.(Gly12Cys) at the protein level (also known as G12C). The presence of this specific KRAS variant in this sample indicates that this patient may benefit from G12C-targeted therapy (8).

No currently actionable variants were detected in the EGFR gene in this patient's tumour sample. Current clinical evidence suggests that this patient has a reduced likelihood of response to EGFR tyrosine kinase inhibitors (1).

BRAF p.(Val600Glu) (V600E) variant NOT detected in this patient's tumour sample.

Any variants of unproven clinical significance detected have been listed in the technical information (10).

The EGFR hotspot regions (exons 18-21) were successfully sequenced to the required quality standards to detect a variant allele down to 5% in a background of wild type DNA; variants in these regions account for 99% of the EGFR variants listed in lung tumours in COSMIC (7a).

The gene regions associated with BRAF p.(Val600Glu) and KRAS p.(Gly12Cys) were successfully sequenced to the required quality standards to detect a variant allele down to 5% in a background of wild type DNA.

Figure 6: An anonymized example gene panel analysis report from a Genomic Laboratory Hub illustrating cross reference details of source tissue biopsy. Source: Authors clinical practice files.

The finding that stage 4 patients are more likely to decline makes clinical and biological sense but there may be additional scope to refine further.

What is also interesting about this group of stage four patients was that 75% of them had a PS0-2 at that time and would have been considered for radical treatment by our oncologists. On this basis we proposed a form of rapid gene testing be reflex requested and carried out on tissue from this group to prevent missed treatment opportunities. Half of our patients present with stage 4 disease, in keeping with national data. The proportion of stage 4 lung cancer presentations has increased since the COVID-19 pandemic. Some estimate the proportion of stage 4 presentations to have risen to around 75% of cases during the pandemic (134-136).

An options appraisal to assess potential testing methods for EGFR single gene somatic testing at the time of study highlighted the Idylla EGFR test as the best fit assay for in-house testing given our available resources of time, laboratory space and biomedical staff expertise. Therascreen and Cobas were excluded as potential testing option because DNA extraction in a clean facility was required and to make financially viable batching would be required. The Idylla system had the advantage of being able to do a test for one patient when they needed it without any change in cost. Furthermore, the Idylla system did not require nucleotide extraction nor molecular grade dedicated clean space. Attempting a real-world testing regime by performing the Idylla test just after NGS slides were sent for testing on a prospective basis meant we were able to describe a turnaround time in house for EGFR results of 3.8 calendar days.

We then moved onto testing formalin fixed paraffin embedded tissue of malignant lung tissue samples in the cohort in which requesting a DNA panel was indicated. In this way we hoped to look at concordance of Idylla rapid PCR testing and whether the outcomes were concordant with the DNA panel NGS. At that time this meant all non-squamous, non-small cell neuroendocrine carcinomas were tested. The study took place at a time before RNA sequencing had been introduced as a means of detecting gene fusion cancer drivers. The samples used had been archived for storage following standard of care diagnostic and theranostic use; we used tissue left over following DNA NGS. There were 102 samples tested from 96 patients (some patients had more than one biopsy sample, for example when looking for resistance mutations in patients relapsed on tyrosine kinase inhibitor therapy and some had more than one site sampled for staging purposes). We found a concordance that was acceptance for clinical use at 96.39% (confidence interval 92-100%).

There were 11 NGS tests that failed to produce findings (failure rate 10%) and the subsequent Idylla test was able to produce a valid result in 9 of those cases that failed, including 2 positive findings of L858R mutations. Idylla failed to produce a valid result in one case where NGS had reported an exon19 deletion because there was insufficient remaining tissue for Idylla. A minimum 10% tumour nuclear content is recommended by the manufacturer. The prior NGS test had consumed 60µm of tissue, as is standard for our reference laboratory. The Idylla test in contrast consumes one 5µm thick tissue section. We discuss in our paper the importance of judicious use of valuable diagnostic tests and how pathologists, being guardians of the sample and being able to assess cellularity, are the healthcare professional best placed to decide what tests should follow and using the most appropriate method for the sample size. This is also reinforced by pathologist attendance at MDT where relevant clinical information around urgent of the patient circumstances are known.

Judicial use of limited tissue specimens is of clinical concern in thoracic pathology where patient specimens are often very small, particularly mediastinal lymph node aspirates and bronchial biopsies.

We go on to discuss potential issues around integration and implementation of a parallel testing protocol. Idylla EGFR test include 52 known actionable variants within the EGFR gene exons 18-21 but it will not detect rare or novel point mutations or small insertions or deletions. The Idylla mutation test covers around 90% of actionable mutations in EGFR as a proportion of what occurs. Oncologists are wary of the possibility of a rapid negative PCR report for EGFR mutation followed by a positive finding on a subsequent NGS report and how to explain that sufficiently well to patients within time-constrained NHS outpatient clinics. This is an understandable concern but one which could be overcome by pathologists reporting, "Await NGS report", instead of a negative PCR.

As a result of this EGFR study, we asked ourselves the question whether these findings in the lung cancer domain had any relevance for other solid tumour types. Melanoma was identified as a potential candidate disease where patients with a high burden of disease are known to deteriorate rapidly. Our next paper examined concordance of the Idylla BRAF test with NGS findings and looked at tissue consumption issues.

Since the EGFR work was published the repertoire of genomic biomarkers that are clinically actionable has increased. We now perform RNA sequencing, in addition to DNA sequencing, which consumes 90µm of an FFPE tissue block to give information about gene fusion drivers of malignancy including ALK1, ROS1, NTRK 1,2 and 3, RET and MET 14 skipping lesions. The (Genomic Laboratory Hubs) GLH also reports on EGFR structural rearrangements. This has led to further work being undertaken in house to look at whether this is a good use of valuable tissue. So far, we have determined that a third of RNA NGS tests fail because of poor quality RNA. A failure rate of 2% was quoted by a multi- institution European study of the new Idylla Gene Fusion cartridge. We are in the process of doing a concordance study using archived samples from the audit cohort. Preliminary results show that this method could be successfully used as a salvage technique in failed RNA sequencing cases. We were able to demonstrate 18 of such failed tissue samples could yield a valid gene fusion result using the Idylla technology (unpublished).

Impact of the Study

This publication has received much attention and critique. The findings were invited for presentation in the UK, Europe and the United States of America at companion diagnostics and pathology conferences. Further, the contents formed the basis of an online Webinar with a global audience of up to 200 people. Much of the discussion about the paper focusses on patient benefits, particularly time to treatment and the subsequent impact on progression free and overall survival in stage 4 lung cancer patients. It has been shown that survival benefits from commencing tyrosine kinase inhibitors in a setting of EGFR mutated adenocarcinoma is lost if started after conventional chemotherapy +/- checkpoint inhibitor therapy, which may be used by some oncologists as a 'holding' mechanism whilst waiting for NGS reporting in stage 4 patients at risk of deteriorating (52). Smith, *et al,* report an apparent median apparent survival (AS) of 672 days in a group of stage four lung cancer patients who switched therapy following identification of an actionable driver mutation (Group B) and this is comparable with the group who did not switch to TKI therapy (Group C; AS= 435 days) (52). See figure 7.

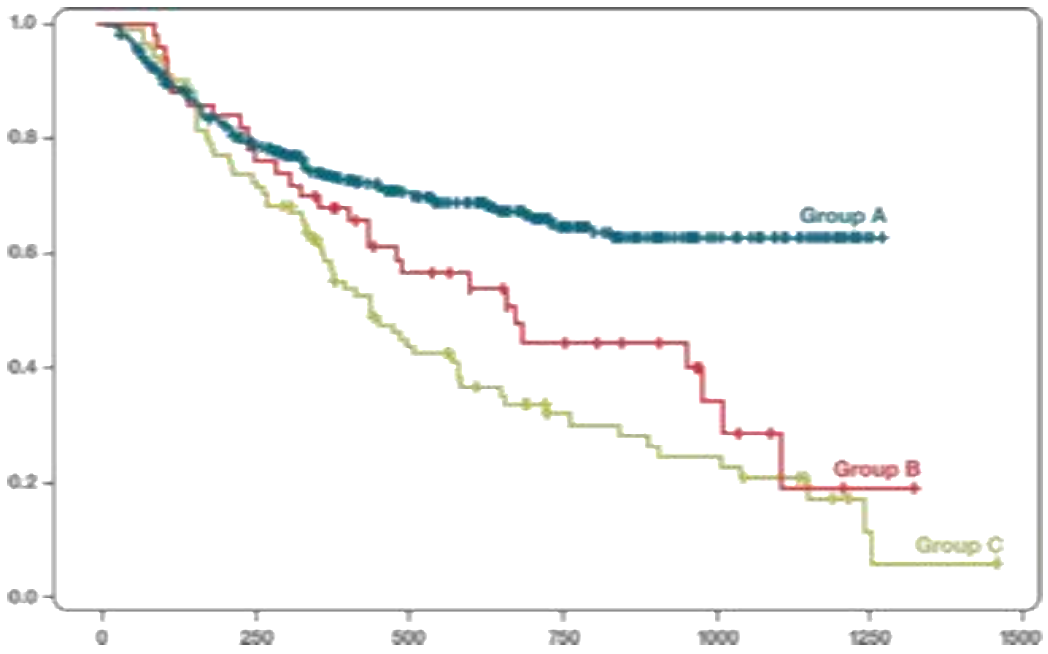


Figure 7: Source: Smith RE et al. (2022) J Clin Oncol. 40(16_suppl):1530. Median apparent survival among different patient groups demonstrating effect of genomic profiling directed treatment decisions.

Their control group (A) who received first line TKI therapy after genomic profiling had a 22% higher probability of survival than group B and a 35% higher probability of survival than group C. Smith et al were using a rapid NGS sequencing technique assessing a small panel of gene that generates results in 1-3 days (52). Not all authors agree with the findings of Smith, *et al* (52). Almeida, *et al*, conducted a similar retrospective study of outcomes when switching from platinum doublet chemotherapy to TKI after somatic EGFR mutations were reported and found no difference in survival (137). However, this study analysed clinical data from just 31 stage four lung cancer patients compared with 525 patients in the Smith study. Further the Almeida group did not use a control group. There was potential for widespread impact on local clinical practice for the benefit of lung cancer patients based on the findings of our study to support need for change. This opportunity was not deemed a priority for local public-funded health services however and did not materialize.

iii)Improving care of melanoma patients through efficient, integrated cellular- molecular pathology workflows using tissue samples with low tumour nuclear content.

The overarching objective of our research was to look at whether we could extend the findings from our EGFR study and improve turnaround times for reporting somatic mutations in advanced melanoma. We recognise that those patients with aggressive malignancies such as malignant melanoma and those with a high tumour burden need rapid testing for somatic BRAF mutations to ensure benefit from MEK inhibitors and /or immunotherapy (65, 138). Malignant melanomas harbour BRAF mutations in around 50% of cases and the vast majority result in an amino acid substitution at position 600, most commonly V600E (139). Malignant melanoma is one of the least common types of skin malignancy but one that is responsible for the greatest number of deaths compared with other skin cancer types (140).

Three questions were raised for analysis in our paper regarding use of rapid PCR for testing melanoma samples for BRAF mutations. Is the test fit for purpose in our hands and what is the concordance with NGS DNA panel findings? Further, we were aware that the manufacturer recommendations stated a tumour nuclear content (TNC) of 50% was required for valid testing and to comply with instructions for use. We wanted to assess whether it was possible to produce a valid BRAF mutation result using FFPE tissue samples of melanoma with less than 50% tumour nuclear content (TNC) using the Idylla system. Evaluating TNC relies on a semi-quantitative assessment of the number of viable tumour nuclei as compared with background 'wild-type' non-malignant normal cell nuclei, be they stromal cells in connective tissue or lymphocytes that make up a lymph node. There is the potential for overestimating the amount of tumour present if one relies on tumour area particularly in a setting of lymph node metastases (141). The concept of tumour nuclear content estimation is illustrated in figure 8.

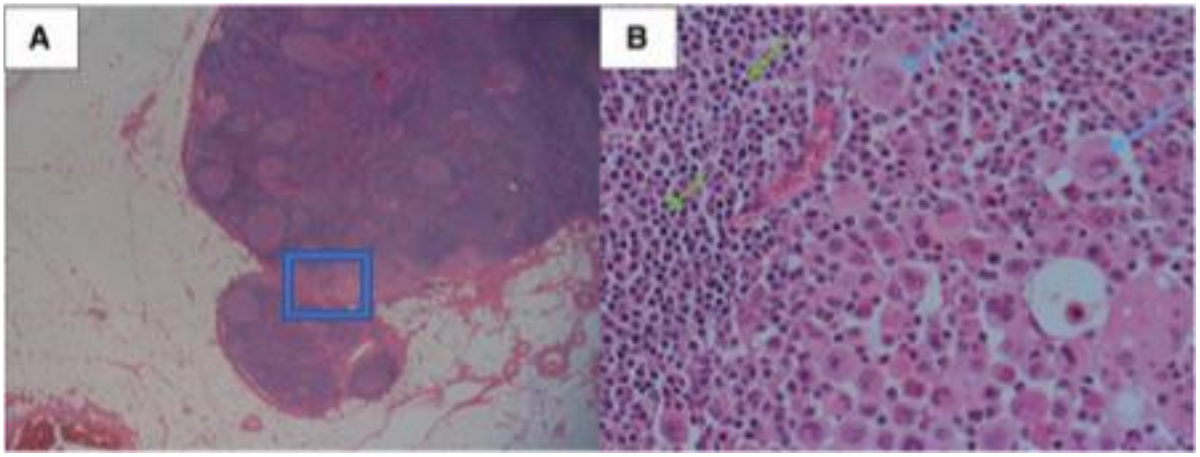


Figure 8: Estimating percentage tumour nuclear content.

Photomicrographs of metastatic malignant melanoma to a lymph node illustrate how wild-type nuclei of lymphocytes can drown-out a mutation signal on whole section tissue DNA extraction without microdissection. Source: Finall et al, 2022(8).

One hundred patient samples were prospectively identified for testing following completion of NGS testing according to standard operating procedures and guidance in Wales at that time. Those cases with less than 50% TNC were macro-dissected using a non-water-bath based method on a molecular grade clean microtome guide plate and sterile scalpel blade. A corresponding haematoxylin and eosin-stained tissue glass slide marked by a pathologist was used a reference to guide dissection. TNC was below 50% for 64% of melanoma cases and only three of these cases failed to produce a valid result. The TNC was estimated at 10%, 30% and 40% and all had Cycling Quotient (CQ) values below 40. We found that of ten cases with a TNC below 10 all but one produced a valid result using the Idylla system for BRAF testing. We suggest that the TNC 50% threshold issued by the manufacturers is unreasonably high based on our data. Adhering to the instructions for use would mean that more than half our patient cohort would have been classified as ineligible for testing using Idylla from the outset. Our findings are in keeping with other studies that have assessed minimum percentage TNC for testing for BRAF mutations in somatic tissue testing using the Idylla platform (142-144).

In addition, we found a BRAF mutation test concordance of 95% using Idylla when compared with NGS. The primer sets within the BRAF testing system include actionable variants V600E/E2/D or V600K/R/M. These are all actionable variants in BRAF that can be treated with MEK inhibitors. Two of the BRAF mutations detected by NGS were variants of uncertain significance and missing these findings would have had no impact on patient management (145). A variant in exon 15 of the BRAF gene was seen in one patient, outside the primer set available in Idylla. This variant has been shown to respond to MEK inhibitors in isolated cases reports published in the medical literature (146, 147). The other two variants have been shown in case reports not to respond to MEK inhibitor therapy (148, 149). In conclusion, just one patient in 100 may have missed out on a treatment option if Idylla had been used as the only method of BRAF mutation detection in our melanoma cohort.

There are real-world logistical reasons for wanting to use a rapid detection method for BRAF mutation in melanoma patients and also in lung and colorectal patients where BRAF mutational analysis may be required. Some of these points we have touch upon already; Improved turn-around time in patients with a high burden of disease at risk of rapid deterioration and limited amounts of tissue prior to testing (149). Benefits of in-house testing using an automated PCR system include the ability to accurately macro-dissect tissue specimens for TNC% enrichment to ensure testing success. Many centralised GLH's performing somatic genomic testing for cancer patients do not have access to trained tissue pathologists to support such pre-analytic quality metrics and some may not have access to the original diagnostic cellular pathology report. National guidance recognized the importance of integrating cellular and molecular findings in one report to avoid confusion and clinical incident (108).

Since our paper was published, NICE guidance has been updated to recognize the role of immunohistochemistry (IHC) in detecting V600E mutations rapidly in a context of malignant melanoma (49). Immunohistochemistry is widely available for use in cellular pathology laboratories, including in resource restricted countries, and allows for direct reporting with cellular morphology within 2 days with a high degree of sensitivity and specificity (49, 144, 150). Care must be taken in interpreting the assay in metastatic melanoma tumour deposits and when the lesion is heavily pigmented (151). Use of well-defined scoring criteria can help

reduce interobserver variation between pathologists interpreting the assay in difficult cases (151). The BRAF V600E IHC assay has also been shown to be reliable in cytology specimens (152). However, it should be noted that the BRAF V600E assay will not detect less common variants in BRAF that can be present in as many as 10% of melanoma patients (150). For this reason, one might consider reserving use of the V600E IHC assay for urgent cases only where treatment decisions need to be made rapidly (150).

iv)Inadequacy of PCR genotyping in advanced non-small cell lung cancer. A counter perspective.

This correspondence piece was written in conjunction with colleagues in England both in private and NHS facilities well versed in reporting EGFR mutations in non-squamous, non-small cell lung cancer patients (NSCLC) (9). The paper is a response to a case report of one patient where a rare mutation in the EGFR gene was detected using circulating tumour DNA (ctDNA), also known as 'liquid biopsy'(153).

Some background information regarding ctDNA mutation detection is required before moving onto the specifics of the manuscript. This method of mutation detection in somatic malignancy relies upon DNA or RNA extraction from a peripheral blood sample (154). There is significant clinical promise for early detection of malignancy, identification of specific genomic alterations to guide oncologic treatments and monitoring of disease course (154, 155). Liquid biopsy has the advantage of being relatively non-invasive and has the potential provide information regarding the whole tumour genomic landscape without being compromised by tissue sampling heterogeneity (155-158). However, much of the research data relating to use of liquid biopsy comes from studies that are regarded as of low or critically low quality (155). The American Association of Cancer Care Centers acknowledges that there is some confusion in the healthcare professional community about what liquid biopsy can and cannot achieve for patients and that there is a clinical education gap that needs to be addressed to support appropriate use of liquid biopsy in in a clinical setting (154) and others stress the urgent need for standardization of preanalytical parameters for liquid biopsy and testing techniques with detail research and clinical trials (155).

EGFR mutations are only found in <1/5 of NSCLC patients and adenocarcinomas at other sites may harbour EGFR cancer driver mutations (159). NGS sequencing or PCR analysis of EGFR mutation must only be undertaken in the clinical setting of *tissue* biopsy proven primary NSCLC (156). In practice, ctDNA sequencing is of great value in detecting resistance mutations in NSCLC patients receiving TKI therapy with a known diagnosis of pulmonary NSCLC (160, 161). Furthermore, the identification of cancer drivers, particular of lung origin,

using ctDNA has a low sensitivity in early-stage disease as there is little shedding of tumour DNA and/or malignant cells into the systemic circulation (161). CtDNA analysis only has a reliable useful detection rate in late-stage disease (stages 3 or 4) and sensitivity of reliable reporting increases with degree tumour burden from disseminated malignancy (157, 158).
The publication by O'Sullivan*, et al,* highlights a single case of a 63-year-old man with stage four NSCLC whose initial work-up included EGFR polymerase chain reaction (PCR) using the Idylla method in addition to immunohistochemistry for ALK-1, ROS-1 and PD-L1 (153). The patient was referred into their institution for treatment and prepared for cytotoxic chemotherapy (carboplatin-pemetrexed combination) with Pembrolizumab. It is not clear whether the patient received doses of this regime before results from a subsequent ctDNA NGS analysis (using Guardant 360), initiated by their department, highlighted a novel, previously unreported, L757_A755delinsSS mutation in EGFR. The authors state that on the basis of this single case all PCR testing in mung cancer be abandoned. One clear criticism of the paper is that such a general conclusion extends beyond what is appropriate for an n=1, retrospective observational report and may provoke alarm amongst patients whose treatment is based upon EGFR mutations detected by such a modality.
The authors are clear that the novel mutation is not described in the COSMIC (Catalogue of Somatic Mutations in Cancer) online database (162). Further, they also state that the mutation has not previously been reported in the medical literature (153). It would be useful to know if the patients' had a meaningful clinical response to treatment to anti-EGFR TKI therapy with this particular driver mutation. This data is not presented (153).

v)RNA next generation sequencing in the somatic molecular testing of non- small cell lung cancer (NSCLC): Is it time to re-consider testing options for improved patient care?

This paper was inspired by a small audit of lung cancer cases. I reported that 35% of samples sent to a centralized genomics laboratory failed to produce a valid RNA sequencing report.

Background

All patients with NSCLC cases are discussed at multidisciplinary team meetings (MDT) in secondary care practice within the UK. These meetings comprise consultants with direct clinical care responsibilities for patients with the radiologists, oncologists and pathologists to sense-check diagnostic information and correlate with the clinical context of each case (72, 109). This process ensures each patient diagnosis is correct and informs the best management plan for each patient. Predictive molecular biomarkers are also needed to determine best treatment options (19, 163).

Knowledge of programmed death ligand-1 (PD-L1) expression in malignancy, as assessed by immunohistochemistry, in addition to somatic molecular biomarkers Kirsten rat sarcoma viral proto-oncogene (KRAS), V-raf murine sarcoma viral oncogene homologue B (BRAF), epidermal growth factor receptor (EGFR) analysis by DNA NGS and gene fusion events can predict response to novel targeted oncological treatments.

Immunohistochemistry for anaplastic lymphoma receptor tyrosine kinase (ALK-1) and ROS proto-oncogene tyrosine-protein kinase (ROS-1), and neurotrophic receptor tyrosine kinase 1, 2 and 3 (NTRK1/2/3) (by pan-TRK) are often undertaken by cellular pathologists as a protein expression proxy markers for underlying genomic fusion events because the stains are widely available, inexpensive and reported rapidly(164-167). These biomarkers may also be reported by analysing RNA sequence for major structural rearrangements in addition to RET proto-oncogene, receptor tyrosine kinase (RET) and skipping lesions in exon 14 of the MET proto-oncogene receptor tyrosine kinase (MET) (24, 168). Fluorescence in situ hybridisation can also be used to detect fusion events effecting these genes but is labour intensive and time consuming (103, 169, 170).

RNA sequencing for gene fusions is preferred over DNA sequencing because the sensitivity is

increased as large intronic regions of DNA which interfere with bioinformatic alignment and effective analysis are removed in the biological process of splicing to form mature messenger RNA transcripts (171-173).

Aims and Objectives

The aim of this study was to ascertain whether this finding was typical of centres elsewhere in the UK using a hub-spoke model of testing and to explore potential reasons for RNA sequencing failure that could be improved. The focus for improvements would be limited to pre-analytic considerations as the RNA sequencing assay was performed in a genomic hub external to the source material. The analytical and post-analytical components of RNA sequencing are, therefore, are beyond the control of cellular pathology laboratory staff.

Findings

Failure rates for RNA sequencing reported in the medical literature vary. There are papers which quote similar failures rates to that experienced in our centre in the region of a third of cases failing (174, 175). Others report better rates of successful reporting RNA sequencing. Some of the more favourable data appears to be skewed, however, by removing cases where yields of RNA measured by fluorometric methods are insufficient to yield reliable results or by repeated testing (176, 177). Those reports of better rates of success appear to be concentrated about cytological studies where the nucleotide extraction is from samples that have had minimal fixation in 10% neutral buffered formalin (176, 178, 179).

Pre-analytical considerations that affect RNA sequencing outcomes include specimen type, time to fixation and the type of fixation method used (if any), storage duration and tumour nuclear content within a tissue/cytology sample (179). Ramani, *et al,* report an overall success rate of 90% for their somatic RNA sequencing using Oncomine ThermoFisher assay which is designed to detect 51 possible gene fusions using solid tumour samples from multiple body site with many different diagnoses. They only tested samples where there was a minimum of 20% viable tumour nuclear cellularity or 300 cells (179). These findings contrast with our experience where we use a minimum of 100 viable cells as the lower threshold for test initiation and only record the tumour nuclear content percentage (10). RNA sequencing at our reference laboratory is not repeated but a ‘salvage’ pathway initiated which involves using the remaining tissue slides for FISH for ALK-1, ROS-1 and NTRK 1. NTRK, 2 and 3 FISH will

only be performed if there is enough tissue remaining to do so.
Furthermore, all RNA sequencing tests were repeated in the Ramani study, if an initial failure was experienced. Their results showed an equally yield of results for tissue core biopsies and cytology specimens (179). There was an increased fail rate for RNA extraction using resection specimens and archived paraffin embedded tissue more than 2 years old (179).

Fixation in 10% neutral-buffered formalin can cause direct damage to RNA nucleotides but is required to prevent tissue degradation as part of standard processing procedures in cellular pathology laboratories (180-183). Some author find that formalin fixation at low temperatures can reduce RNA damage (184). However, snap frozen and fresh tissue specimens are well known to yield better quality (longer nucleotide length) and quantity of RNA compared with fixed counterparts (185, 186).
Cytology specimens prepared into cell blocks with minimal formalin fixation or use of methanol as an alternative have also been shown to give a higher rate of RNA sequencing success (178, 187). It would not be possible to more to comprehensive fresh frozen tissue analysis in histopathology without a major overhaul of interdepartmental logistics (181). We would need an increased number of cryostats for frozen tissue examination prior to sectioning for NGS and these are much larger and more expensive than standard microtomes. There would also need to be an investment in freezer storage space, automated tissue chillers, bench space and staff training (181). Alternatives to formalin fixation are available, including use of PAXgene solution but this is prohibitive based on costs (188). It is also possible to vacuum pack reception specimens to run formalin free surgical theatres but this move is mainly based on health a safety issues surrounding use of large volumes of formalin solution (189, 190). Vacuum packing tissue specimens may be associated with improve RNA yields for sequencing (189). One might argue that fresh tissue cores should be snap frozen and sent directly to the sequencing laboratory but this approach, whilst potentially improving nucleotide extraction would leave doubt as to the tissue diagnosis and tumour nuclear content, if tumour is present. Clearly one would be able to produce a sequencing report in such cases but one would not know whether it was a false negative without prior pathologist examination. Tumour nuclear content (TNC) estimation is an important consideration prior to sequencing(141). Ramani *et al,* only proceeded to test on cases with a minimum of 20% TNC. For TNC estimation to be accurate, competency training is recommended by CAP to reduce interobserver variation and to ensure an appreciation of the influence of background wild-type normal tissue cell nuclei,

particularly in the presence of dense chronic inflammation (191). Assessing tumour area will underestimate the diluting influence of numerous lymphocytes as these cells are composed predominantly of nuclei compared with malignant epithelial cells whish often have more cytoplasm (191). See figure 9.

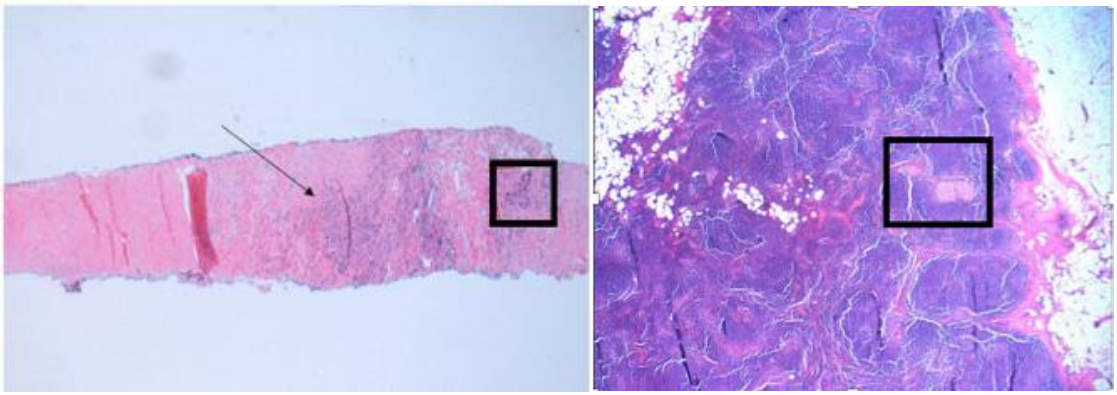


Figure 9: Tissue sections showing specimens of apparent large volume to the operator but with little malignant target tissue on examination of the specimens at a microscopic level. Black squares contain malignant cells. Arrow highlights an area of chronic inflammation in a lung core biopsy. Source: Finall, 2022 (10).

The amount of tissue available in the biopsy or resection specimen will directly affect the amount of RNA available for extraction and the likelihood of a successful result(191). RNA (and DNA) sequencing at our external laboratory is a consumptive process requiring in the region of 120µm of tissue sections be sent for a single NSCLC case requiring both panels. This does not compare favourably with the Idylla system or immunohistochemistry (7).

There are RNA degradation enzymes present in the local environment including the air and all laboratory utensils and surfaces (182). The enzymes destroy RNA within FFPE tissue sections. In most NHS cellular pathology laboratories, FFPE tissue specimens are cut on a microtome in an open room with no special sterile air flows. Thin tissue sections are floated on a water bath prior to mounting on glass slides with the inherent risk of contamination despite efforts to change the water regularly (192). It is not possible to use molecular grade, sterile water in the water baths in a context of a public-funded healthcare system as the high cost is prohibitive. Slides are packed into a non-sterile tissue slide mailers in standard packaging for postage to the external laboratory. These are less than optimal conditions for handling and preserving delicate RNA molecules for sequencing (179). These laboratory procedures may account for why so many tissue samples fail RNS NGS in a hub and spoke model of service provision.

Impact of RNA sequencing failure on NSCLC patients.

Consequences of failed RNA sequencing in lung cancer directly impacts patient care. Missed treatment opportunities and the need for repeat invasive tissue biopsy are obvious examples. Some Genetic Laboratory Hubs (GLH) request upfront slides to be sent if FISH is required to 'salvage' gene fusion analysis.

A multi-centre study of the Idylla gene fusion assay performed across Europe showed the assay to have a failure rate in only 2% of cases of NSCLC(193). Given that GLH RNA sequencing reports a single additional finding compared with the Idylla gene fusion cartridge (structural rearrangements in EGFR), there is a clinical argument in favour of complete replacement of RNA next-generation sequencing assays in favour of the Idylla GeneFusion PCR method (193). This would allow same day results with the tissue diagnosis and consumes less tissue (194-196).

Following on from this paper we have subsequently performed an (unpublished) additional audit of a larger number of patients (n=77) and with similar results (failure rate 32%). We took the 20 cases that failed RNA sequencing and found that we could successfully salvage 91% of them (n-=18). This was despite the tissue blocks already having been used for both DNA sequencing with the inherent consumption of tissue.

Publication Critique

There is limited new data in this manuscript (the identification of a failure rate of 35% for RNA sequencing). However, an extensive review of current evidence served to educate the author as to issues surrounding use of RNA assays in clinical practice and look for ways to improve the service for patients. Knowledge of this real-world failure rate based on a hub- and-spoke model of service provision is extremely important for service monitoring and planning.

Impact of this publication

This paper was published very shortly after implementation of clinical RNA sequencing to assess for gene fusion events in lung cancer in the UK. Its publication highlighted potential shortcomings of the technique ahead of launch for some laboratory hubs in England and therefore presented an opportunity to put appropriate salvage methods in place from the start. The use of gene fusion assays for NSCLC patients also applies to squamous carcinomas with respect to NTRK, and potentially all other solid tumours that fail standard approved treatments, so the impact is wide.

Writing this article was used to support maintenance of an IHC based service for ALK-1 and ROS-1 and to support introduction of panTRK IHC to add to the in-house lung cancer predictive biomarker IHC panel.

vi)Analysis of somatic epidermal growth factor receptor (EGFR) mutation by rapid polymerase chain reaction (PCR) using intra-operative frozen section tissue in early stage, non-small cell lung cancer patients.

This study started just prior to the COVID-19 pandemic in 2020 in the knowledge that Osimertinib would be licensed for adjunct use in the post-surgical setting for NSCLC later that year (83). The aim was to ensure that the EGFR Mutation Test, a rapid automated PCR test for use on the Idylla platform would give the same standard of results as compared with use of FFPE tissue samples (197). The test is licensed for use based on FFPE tissue input, but other groups have verified the assay using other input material such as extracted DNA and cytology specimens (198-200).

There is a single paper referring to the use of frozen tissue input for rapid PCR but as part of a diagnostic work up before surgery and prior to systemic treatment decisions(201) (170). This is the first publication using *intra-operative* frozen section samples to inform adjunct treatment with Osimertinib in the post-operative setting (197).

We designed a prospective comparison study using 12 consecutive patients undergoing intra-operative frozen section diagnosis in our centre. If the pathologist determined a diagnosis of adenocarcinoma or non-small cell carcinoma at microscopy, with sufficient amounts of malignant tissue (TNC >20%) and consequent lobectomy and lymph node dissection, the patient sample was included for study. A sequential frozen section was taken from the cryostat immediately following completed diagnostic frozen section requirements and an EGFR test initiated on the Idylla platform.

Data regarding cycling quotient (CQ) was recorded with TNC%. Tissue immediately adjacent the area examined for frozen section was embedded for processing onto an FFPE tissue block. This allowed for the closest similar tissue section to be used as FPPE tissue input into the EGFR Mutation test for most accurate tumour comparison.

We found a statistically significant difference in the CQ value for reports using the EGFR Mutation test between frozen and formalin fixed tissue samples (197). The lower the CQ value to better the quality of DNA, fewer cycles of PCR are necessary to produce sufficient quantity of DNA for analysis. According to the manufacturer instructions for use, a CQ value

of 20 is equivalent to 200ng of DNA (202). The frozen section group had a mean CQ value of 15.6 and the FFPE group a mean CQ value of 23.2 (p value <0.0001).

Impact of the study

Analysis of frozen tissue input to the Idylla EGFR Mutation Test cassettes was verified as being suitable for clinical needs of our patients. The study also highlighted, though not discussed, the complexity and inconsistency around EGFR requesting. The complexity of molecular testing and the need for pathologists to stay up-to-date with the needs of patients with different histological types of lung malignancy and in different clinical settings, combined with a need to reflex request molecular studies whilst reporting, lead to the decision to restrict reporting of thoracic subspeciality specimens to dedicated thoracic pathologists in our unit. Even with this measure there is variation in requesting amongst our patient cohort. Variation in how molecular testing for lung cancer is requested and whether patients have access to relevant genomic testing in a setting of lung cancer exists across the UK and the world (129, 130, 203, 204).

Publication Critique

This was a small study but was limited following unforeseen circumstances around the pandemic. All thoracic surgery in our institution was halted in the early stages of the pandemic. Also, no frozen sections were allowed at all following safety concerns regarding the generation of tissue aerosols as an inherent part if the process of cryotomy (192). After introduction of respirator body suits for the histotechnologists to wear during cryotomy, frozen section examination was re-introduced but thoracic surgical procedures remained low in number as one of the centres two thoracic surgeons was isolating for the duration of lockdowns for personal protection. During this time, our allocation of EGFR mutation cassettes to support a larger study became out of date and reliable testing could not be guaranteed leading to termination of the study.

Not all the patients in our study cohort had DNA NGS prior to surgery. This was standard practice at that time. Before Osimertinib was licenced for adjunct use, only stage 3 and 4 NSCLC patients were liable to need biomarker data from their diagnostic biopsy sample to inform

treatment. The rationale behind not testing all patients was to prevent waste and excess costly testing for patients that did not need it. Clearly, intimate knowledge of the eligibility criteria for EGFR testing at that time was not fully appreciated by all pathologists and some patients did have DNA NGS performed. This information, although not planned to be collected was added to the data collection to see if any inconsistencies were identified.

This comparison, whilst favourable for rapid PCR, is not of sufficient number to be robust. A sample size calculation was not performed. The resulting study was limited by availability of EGFR Mutation test cassettes.

Chapter 3

Integrated Narrative and Discussion

How do the published works contribute to medical knowledge?

Our paper promoting the integration of PCR alongside NGS in NSCLC gives evidence of clinical need for rapid near-patient testing (7). This is important to prevent patients with a high burden of disease at risk of rapid clinical deterioration from missing out on testing and potential treatment. We found that 18 percent of our NSCLC patients with stage 4 (disseminated metastatic) disease underwent rapid clinical deterioration and potentially missed out intervention with a TKI drug. We further described how this cohort were usually fit for active oncology treatment; 75% of the stage 4 patients had a performance status of 0, 1, or 2 at diagnosis (7). Similar findings have been reported by other European centres (205).

To date, patients with a poor performance status (PS3 or 4) would often receive best supportive care and symptom control rather than chemotherapy or surgical intervention (133, 206). Data is emerging, however, that supports active treatment in this population. A recent observational study of patients with a poor performance status (PS3 or 4) and EGFR mutated lung non-squamous, non-small cell carcinoma found an objective response of 56% to first line Osimertinib in this group with a good safety profile (207). Interestingly, Igawa and colleagues also found that performance status improved in a small proportion of patients and that progression free survival was, on average 10.5 months (207). This data is further supported by a recent phase 2 clinical trial showing a response rate to Osimertinib of 63% in patients with lung cancer patients with a poor performance status, over half of whom had brain metastases (208). Furthermore, the clinical trial data showed an improvement in performance status in 63% of this cohort, progression free survival was reported as 8 months and overall survival 25.4 months (208). As such, there appears to be early evidence that molecular testing in the rapid deterioration group in our paper could have an improved performance status, progression-free and overall survival if they had received appropriate targeted therapy.

It is well established that subtype/ predominant growth pattern of lung adenocarcinoma correlates with clinical prognosis (72, 209-213). Solid, cribriform, papillary and micropapillary growth pattern pre-dominant adenocarcinomas of the lung are associated with an increased risk of recurrence and worse prognosis (210-213).

There are recent studies that examine the correlation between molecular drivers of malignancy and their histological phenotypes. Pyo and colleagues compared a large cohort of adenocarcinoma of lung and stratified by histological subtypes (214). They found that ALK-1 fusion drivers were over-represented in adenocarcinomas with a cribriform growth pattern (214) and this has been substantiated by others (215). Abolfathi, *et al* discovered that there is a statistically significant difference in the incidence of EGFR exon19 deletions in between acinar predominant pattern lung adenocarcinoma (present in 6% of cases) and those with a predominantly non-acinar histopathological pattern (2.9%) in a large study comprising 1263 cases (216). A smaller but detailed gene expression study of 31 patients with adenocarcinoma lung conducted by a German group found that tumours from the same patient had both heterogenous morphological patterns and molecular signatures (217). Gene expression analysis showed gene expression signatures inhibiting apoptosis and promoting metastatic behaviour and cell proliferation where present in all the growth patterns of adenocarcinoma except the lepidic growth pattern (217). This makes biological sense when one considers that the lepidic pattern represent an in-situ form of alveolar neoplasia which presents as slow growing 'ground glass' areas radiologically which are frequently monitored radiologically for some time before surgical intervention (218, 219). Whilst the medical literature indicates that associations between molecular signature and histological growth patterns affect clinical outcomes, it remains to be seen what factors are responsible for the rapid clinical deterioration of the patients in our stage 4 group (7). Our comparative analysis of patients who deteriorated rapidly compared with those who did not, showed only a statistical difference in stage at presentation to account for such a difference in clinical outcome. Stratification of the non-mucinous adenocarcinomas into well, moderately and poorly differentiated types showed no differences, and this may be a consequence of small sample size (n=96) (7). To be directly comparable however, the sample samples would need to be reviewed and reclassified based on growth patterns rather than degree of differentiation. See figure 10. Further work with more detailed DNA sequencing and analysis of gene expression data would be required for comparison with some of the findings in the medical literature and this represents a short coming of our work.

Our data suggests a good level of concordance between the Idylla^{TM} *EGFR* test when compared with NGS findings (7). This is in keeping with previous verification reports as would be expected for a CE-IVD approved assay by the Medicines and Healthcare Products Regulatory Agency (MHRA) in the UK (142, 159, 220).

Clinicopathological features	Total (n=96)	Clinically stable patients (n=79)	Patients who rapidly deteriorated (n=17)
Age (years), mean, (range)	72 (41–92)	75 (41–92)	63 (57–83)
Sex p=0.187			
Female	49	43	6
Male	47	36	11
ECOG PS at histological diagnosis p=0.215			
PS0	17	13	4
PS1	41	37	4
PS2	28	24	4
PS3	8	5	3
PS4	0	0	0
Not recorded	1	0	1
Stage at histological diagnosis p=0.049			
I	11	10	1
II	5	5	0
III	17	16	1
IV	47	33	14
Metastatic recurrence	16	15	1
Specimen Type, n (%) p=0.534			
Lung resection	7	7	0
Lung core biopsy	36	30	6
Bronchial biopsy	5	4	1
Pleural biopsy	2	1	1
Lymph node core biopsy	14	9	5
Skin or soft tissue biopsy	6	6	0
Bone	3	3	0
Pleural fluid	4	3	1
EBUS	16	13	3
FNAC lymph node	1	1	0
Other (pericardial fluid, liver and breast)	2	2	0
Smoking status, n (%) p=0.115			
Never smoked	7	4	3
Ex-smoker	46	41	6
Ex-smoker, now vaping	3	2	1
Current smoker	32	29	3
No recorded	8	4	4
Histological diagnosis, n (%) p=0.169			
Adenocarcinoma, poorly differentiated	48	40	8
Adenocarcinoma, moderately differentiated	24	18	6
Adenocarcinoma, well differentiated	8	8	0
Adenocarcinoma mucinous or enteric-type differentiation	5	2	3
Adenosquamous	2	2	0
Large cell neuroendocrine carcinoma	1	1	0
NSCLC, NOS (non-SCC/ p40 negative)	2	2	0

Figure 10: Illustrating comparison of patients who deteriorated rapidly compared with those who had stable disease in a setting of non-squamous, NSCLC.

Red indicates data showing a statistically significant difference ($p<0.05$). Green highlights the performance status (PS) of these patients as an indication of eligibility for treatment intervention by accepting criteria at the time of publication.

EBUS, endobronchial ultrasound guided cytology; ECOG, Eastern Cooperative Oncology Group; FNAC, fine needle aspiration cytology; NOS, not otherwise specified; NSCLC, non-small cell lung cancer; SCC, squamous cell carcinoma.

We found that there is variance in reporting of turnaround times for NGS depending upon how one defines the starting and finish end points. We argue that turnaround times for NGS should include slide preparation and transportation time to more accurately reflect patient experience of the pathway and on-going proliferative biology of malignant neoplasia. Time taken for transporting specimens to genomic laboratory hubs was recently highlighted in a UK -wide standard as an opportunity for improvement in turnaround time for reporting cancer gene panels (129). Time from sending the material from our centre was 17 calendar days. When one also includes the cellular pathology laboratory time to prepare tissue sections, this turnaround time for NGS increases to 23 calendar days, and this finding is in keeping with other centres' experiences (129, 221-224).

Polymerase chain reaction-based single gene testing has limitations in terms of breadth of genes assessed in one test and also the ability to analyse only known gene mutations/ fusions identified as being clinically significant in prior research (9, 153). The Idylla ^{TM} EGFR test contains primer sets designed to capture the majority of known actionable genomic alterations and all those that feature in global, phase 3 clinical trials assessing targeted treatments in NSCLC (202). Around 90% of pathogenic variants in EGFR in NSCLC are either exon19 deletions or L858R mutations in exon 21 (225, 226). A near infinite number of possible *EGFR* variants could be identified by NGS, in contrast to PCR, some of which will be entirely novel and others which will have limited evidence of response to TKIs (227-229). There is evidence an exon 19 specific EGFR mutation is associated with enhanced progression-free (PFS) and overall survival (OS) compared with the other most common classic EGFR mutation, L858R (229). Furthermore, it has also been shown that tumours habouring a high (>70%) mutant allele frequency have both longer PFS and OS independent of mutation type and other factors such as stage, age, gender and smoking status in multivariate Cox analysis (229). This makes biological sense when one considers that lung adenocarcinomas are heterogeneous, both morphologically (230) and on a molecular basis. (225, 231, 232). It is possible that only a portion of a patients' tumour is driven by a particular mutation, and this will be reflected in the variant allele frequency in a well sampled lesion. Small biopsies, therefore, may not be representative of the molecular composition of the entire lesion (156). Varant allele frequency (VAF) is a datapoint provided by NGS and can be inferred by analysis of the amplification curves in real-time, quantitative PCR present in Idylla cartridges also; a low CQ value indicates a higher VAF (233-235). There is no apparent benefit of one method over another to determine variant allele frequency and give information to oncologists regarding prognosis.

Identifying novel and rare *EGFR* variants is a worthwhile pursuit to allow patients to access to TKI treatments and clinical trials where available. Parallel testing of rapid Idylla™ and NGS at the same time, where the amount of neoplastic tissue allows, would appear to harness the benefits of rapidity and breadth in those patients where there is clinical urgency and this clinical approach is advocated in may major centres (233, 236-238). The wider menu of PCR-based Idylla™ tests allows for detection of all the current actionable genomic alterations in lung cancer including *KRAS* and *BRAF* in addition to all the gene fusions (143, 159, 239, 240). These additional tests are also CE-IVD marked for clinical use and have the advantage of much less tissue requirements for successful detection. The gene fusion cartridge would be a very useful addition to

the histopathology repertoire of tests as it has utility in thyroid papillary and follicular carcinoma (RET), papillary carcinoma of the kidney (MET) and all solid malignant tumours that have progressed and failed standard evidence-based oncology treatments (NTRK 1,2,3) (239, 241-243). Additional merit is also to be highlighted from the difficulties in RNA NGS and emerging data of a high failure rate with RNA sequencing as detailed in the article published in the Journal of Molecular Pathology (10). The importance of this publication is to highlight the impact of RNA sequencing failure on the downstream wait for data regarding oncogenic fusions in *ALK-1, ROS-1, NTRK* from FISH. This adds an additional 2-3 weeks to the biomarker pathway for gene fusion identification. The suggested solution to this problem is to use the Idylla™ GeneFusion test cartridge with its comparatively much lower failure rate of 2% (244).

Additional work looking at the utility of the Idylla *EGFR* assay using fresh frozen tissue for intra-operative diagnosis showed good CQ values, inferring larger quantities of DNA are available for analysis in the Idylla™ *EGFR* test (197). This method was shown in a small cohort to be fully concordant with the fraction of samples that were used in NGS. The importance of this work is to demonstrate the safe potential to save NHS resources by performing a single gene test (*EGFR*) rather than multi-gene panel NGS at a higher cost (245). There is usually no need for a rapid turnaround time in surgical candidates with NSCLC as they have had their tumour removed at lobectomy and are early-stage cases with no disseminated disease (197). *EGFR* mutation status is required in a surgical setting to highlight patients for eligible for adjunct Osimertinib treatment (246-248).

All the information from our publications can be summarised in a decision tree aimed at educating medical and nursing colleagues about the role of the pathologist in patient care and

diagnosis in addition to supporting histopathology colleagues working in this complex, and rapidly changing field. See figure 11 below.

The immediate impact of the published works on patient care to date has been small but significant to individual patients. Occasionally an oncologist will contact out department directly to request single gene testing for urgent cases of melanoma and NSCLC. Some tests have yielded actionable mutations in EGFR in lung cancer and directly informed first line treatment. It has been shown by a group in the US that survival benefits from TKI therapy in patients with NSCLC may be lost if the patient is not treatment naïve (52). Put another way, if this patient had started chemotherapy because the oncologist was worried about rapid clinical deterioration, she would not have benefited from switching to a TKI later on when the NGS report was available (52). An additional benefit for patients prescribed TKI therapy is the ability to take an oral agent at home rather than attending the day-unit on the hospital site for IV administration of platinum- based chemotherapy, and reduced levels of anxiety whilst waiting for somatic NGS data to be reported.

Outline the themes giving the works their defining coherence

Technology

The key themes linking all the published works is the use of automated PCR technology adjuncts to NGS for rapid somatic tumour analysis, where clinically appropriate. This thesis brings together a series of work to show that adjunct use of Idylla assays could assist in highlighting additional patients for treatment with TKIs who might otherwise deteriorate within the time frame for NGS reporting. For malignant melanoma, this work has since been made largely redundant by the declaration from the National Institute for Health and Care Excellence (NICE) regarding Dabrafenib treatment, that IHC for BRAF V600E should be used as a first line approach to BRAF testing in advanced melanoma (stages IIC to IV)((49). NICE indicate that BRAFV600E IHC rarely produces false positive results but acknowledge that a back-up method may be required to confirm negative cases to cover the possibility of lesions having V600 mutations with a different amino acid substitution. The NICE committee also acknowledge the potential for this guidance to reduce variation in practice across the UK due to the universal access to in-house immunohistochemistry by reporting cellular pathologists (49).

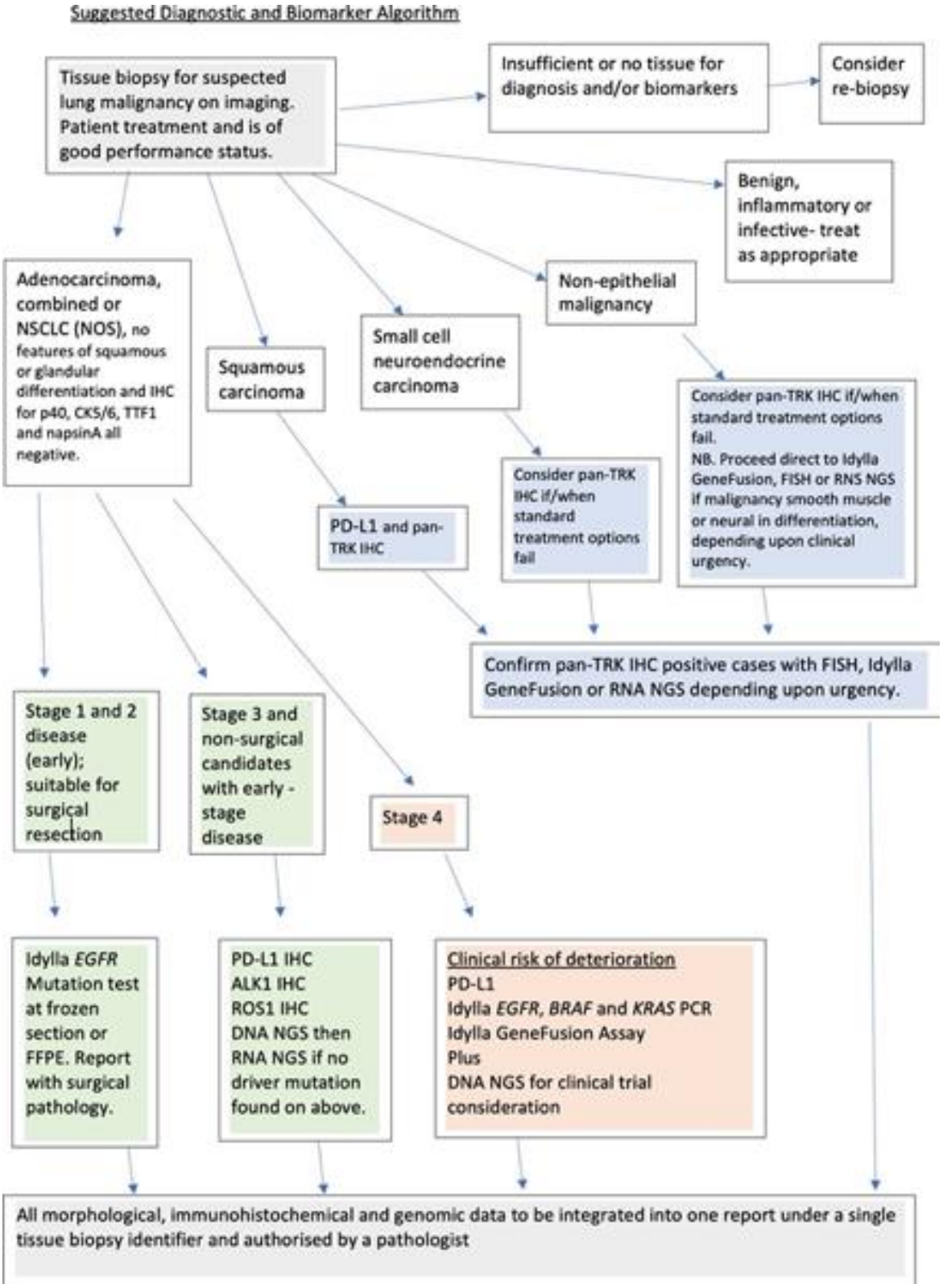


Figure 11: Clinicopathological decision tree for patients with a suspected lung cancer diagnosis.

Our research group have highlighted subgroups of patients who can benefit from this rapid automated PCR technology in a lung cancer setting and shown the utility of the technique in malignant melanoma where there is less than 50% tumour nuclear content. Further, given that Osimertinib has been licensed for use in as an adjunct in the post-surgical treatment of incompletely resected adenocarcinoma of the lung we investigated the use of frozen samples at the time of surgery. In addition, the ethical considerations around choosing the right test at the right time are considered in the Clinical Ethics paper published with the help of Prof. Jones. This concept is further explored in a case report response that dismisses the potential input of PCR-based genomic methods in patient care (9).

Patient-centred care

All the works are written with a focus on patient care and highlight areas for potential clinical improvement that could be influenced by histopathologists if we were appropriately empowered in tissue stewardship. The papers discuss the decision making around best use of limited tissue samples, which patients might benefit from rapid testing and provide evidence in support of the proposed algorithm in figure 11.

Role in co-authored works

My role in the co-authored works was to initiate clinicopathological verification of the Idylla™ technology and liaise with Biocartis to gain loan access to Idylla instruments and consoles plus sufficient testing cartridges to perform the studies. I conceived design of the studies and wrote study protocols for review by the Joint Study Review Committee at Swansea Bay University Health Board, prior to start of the work. The studies were all classified as service development projects due to lack of randomisation and clinical treatment intervention. The committee also assessed that the findings would not be generalisable to other laboratories. I helped to collect the data, analysed the data and wrote the published works in collaboration with colleagues in all but one of the papers; the manuscript entitled, "Inadequacy of PCR genotyping in advanced non-small cell lung cancer: EGFR L747_A755delinsSS exon 19 deletion is not detected by the real-time PCR Idylla™ EGFR mutation test but is detected by ctDNA next generation sequencing and responds to Osimertinib. Sub-title: The apparent "Inadequacy of PCR genotyping in advanced non-small cell lung cancer": A counter perspective" (Appendix E). I also responded in writing to correspondence and feedback forwarded to me by the Journal of Clinical Pathology regarding use of the Idylla EGFR test in lung cancer.

How were the published works tailored for publication?

The first publication regarding the medical ethical issues surrounding investment in precision medicine was written as a review of the literature with particular focus on somatic tumour testing. We addressed issues around autonomy, beneficence and non-maleficence relating to genomic testing. Professor Jones at Swansea University added to and modified the manuscript according to her detailed knowledge of medical ethics. The ethics paper and case experience motivated my involvement in the following papers assessing adjunct testing with rapid fully automated systems. The use of Idylla is proposed as a "belt and brace" adjunct to NGS to prevent patients slipping through the precision medicine net.

The manuscript regarding *EGFR* testing in lung cancer was initially conceived when *EGFR* was the only actionable somatic DNA-based biomarker target relevant to palliative treatment in NSCLC. GLH RNA sequencing became available afterwards. The concept was to perform single gene test using Idylla^{TM} alongside NGS in all patients until our allocated 100 cartridges were exhausted. The *EGFR* testing cartridges were supplied by Biocartis for free on a researcher-initiated study basis, meaning Biocartis staff were not involved in the analysis or write-up of the work. Biocartis merely ask that their material contribution be acknowledged in publication. Designation as a service development project means exemption from NHS research ethical approvals. We were not directed to seek permission from the GLH to use NGS-based information from the patient record as a comparator with Idylla^{TM} for lab verification purposes. Verification of new assays in our laboratory testing repertoire is a standard requirement for UKAS accreditation (ISO standard 15189) even with respect to in-vitro diagnostic tests CE-marked by the UK Medicines and Healthcare Products Regulatory Agency (MHRA).

We used patient samples on an all-comers basis and this included patients from three different hospitals in our locality. Idylla^{TM} testing was automatically initiated following pathologist reflex requesting of NGS.

Strengths and Limitations of the Published Works

The piece regarding medical ethics of genomic testing in solid tumours written with Prof Jones (6) and the manuscript about RNA-based sequencing (10) are based in large part on a review of the literature, save for accounts of personal experience and audit data on RNA sequencing failure rates. The strength of the RNA sequencing manuscript relates to its importance in highlighting shortcomings of an expensive and time-consuming assay at a time of particular resource stress in the NHS (249). There is evidence that the number of diagnostic tests required in the NHS has increased from 800K per annum to over 1.8 million within the last 10 years, but NHS funding and staffing have not kept pace with such increases in demand (113, 250). Any proposal to reduce financial and staffing impact on the NHS whilst maintaining or improving care excellence should be welcomed.

The response letter to the case report published by the London group has strength in the wide range of voices from across the UK united in opinion but again is limited to reviewing evidence already published.

The information from the melanoma study was based on a large cohort of patient samples and dual testing on the Idylla^{TM} platform. The data is of great benefit to clinicians using the system with samples below 50% tumour nuclear content in reassurance of test validity and safety. Its limitations are in breadth of discovery and are largely clinically redundant now that BRAFV600E immunohistochemistry is recommended by NICE for testing in late- stage melanomas in view of its rapid, and cost-effective nature (49).

The strength of the study regarding use of the Idylla^{TM} *EGFR* test as an adjunct to NGS in NSCLC is that is provides data to justify the approach centred around patient clinical deterioration. There is limited data that specifically uncovers the detrimental impact of extended turnaround times in care for such patients as described in our paper. It is limited in its robustness by the relatively small cohort of patients involved. The paper could also be enhanced by inclusion of comparative data looking at progression free- and overall survival of patients following introduction of Idylla^{TM} into the molecular pathology workflow. Such data collection to evaluate performance improvement is not possible without permission from

senior management for clinical roll out of Idylla^{TM} in our laboratory and this is not, as yet, forthcoming.

The frozen section study (197) could have enormous benefits in cost reduction in molecular pathological investigation given that only EGFR analysis is of clinical relevance in an adjunct post-surgical setting in early-stage lung cancer patients. This study is limited by a small sample size due to constraints of the pandemic yet still yielded clear statistical difference in its findings. In this clinical context, only a single gene mutation test, EGFR, is relevant for treatment decisions and would be a cheaper test option compared with panel DNA NGS per patient (239).

Appendix A

Applying bioethical principles for directing investment in precision medicine.

Clinical EthicsVolume 15, Issue 1, March 2020, Pages 23-28

https://doi.org/10.1177/1477750919897380
Empirical Ethics

Applying bioethical principles for directing investment in precision medicine

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Abstract

The concept of precision medicine aims to tailor treatment based on data unique to the patient. An example is the use of genetic data from malignant tumours to select the most appropriate oncological treatment. The competing interests of utilitarianism and egoism create dilemmas for decisions regarding investment in precision medicine. The need to balance the perceived rights and needs of individuals against those of society as a whole is an on-going challenge in the distribution of limited health service resources. There is need for proper planning, organisation and investment into precision medicine to cope with the consequences of both direct-to-consumer and healthcare-directed genetic testing for genetic counselling, therapeutics and diagnostic networks. Consideration needs to be given to providing adequate time and training to allow for meaningful shared decision-making with patients and there is a strong case in support of a hub-and-spoke model to provide rapid, solid tumour genetic mutational analysis to prevent patients missing out on beneficial treatments.

Keywords

Bioethics and medical ethics, genome mapping and sequencing, resource allocation, healthcare economics, genetic counselling, genetics and genomics

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Precision medicine describes the use of biomarker data from companion diagnostics to highlight a subsection of a specific cohort of patients who might respond to a particular treatment.[1] Companion diagnostics can take a number of forms and include testing modalities such as immunohistochemistry and genetic sequencing. Stratified medicine is often used in an oncological setting for patients with late stage malignancies that have a guarded prognosis. Oncological treatments linked to companion diagnostics in this way are novel and expensive. This is an active area of medical research and there are likely to be new drugs provided by the NHS that use companion diagnostics with resulting potential for increased financial burden to the NHS, not only through the cost of drugs, which can run into many thousands of pounds per patient per month, but also through the additional cost of testing large numbers of patients to highlight the subset who may benefit. It is therefore important to consider the ethical principles that underpin investment in precision medicine.

This essay will focus on ethical issues relating to companion diagnostics in cancer patients and consider arguments for and against how to direct investment using opposing consequentialist theories of ethical egoism and utilitarianism.

The first issue in relation to use of companion diagnostics in personalised medicine is who decides whether to test or not. Within the NHS most of these decisions are taken at the multidisciplinary team (MDT) meeting as a collective decision between physicians, nursing staff, oncologist, radiologist and pathologist. These are multifaceted decisions that take into consideration a number of factors such as the histological subtype of cancer, patient performance status, prognosis, social circumstances and patient wishes. This process excludes the patient themselves in a way that is considered counter to patient autonomy, a key bioethical principle underpinning patient care.[2] There is an argument for including patient advocates and or the patients themselves in MDT meeting to allow them to contribute to decision-making.[3] Shared decision-making is an important component of the doctor–patient relationship and such collaboration would be in keeping with GMC guidelines of good practice.[2,4,5] However, shared decision-making, outside of an MDT setting, is a complex process and said to be rarely accomplished in real-life clinical practice.[6] Part of the reason for this may be due to strict research methods and criteria that underestimate when shared decision-making is actually taking place during consultation.[7] There is also a perception that fully informed shared decision-making takes too much time[8] and that evidence for the benefit of that extra time investment may not be readily apparent to the clinician during a busy clinic.[7]

Patient autonomy is a key bioethical principle in medical practice[4,9] and describes patients having the ability to freely choose and determine their fate with independence.[10] The freedom to make a choice based purely on ones' own self-interest would align with Smith's[11] theory that pursuit of self-interest ultimately results in good for all society as if by 'an invisible guiding hand' and this forms the basis for the consequentialist theory of ethical egoism. Patient autonomy requires that patients are in possession of the full facts of their case presented in a clear and understandable way without biased interference by doctors or family members.[12] Patient autonomy is being respected when patients are offered treatment by an oncologist on the basis of full and accurate information. This includes accepting a competent adult patient may refuse treatment regardless of whether it appears sensible to the healthcare professional. This is embodied by Mills'[13] summary of autonomy thus, 'Over himself, over his body, and mind, the individual is sovereign'. There is no evidence in the medical literature around how many patients decline precision medicine in the UK. In the US, however, there is evidence that patients who pay for their own treatment have a reduced compliance rate due to the high costs.[14] Provision of health services on the basis of need rather than the ability to pay, as happens in the NHS, means that this situation is unlikely to occur in the UK. Provision of full and accurate information during consultation needs to be provided in the context of patient power to be sure that decision-making is truly shared.[15]Patients having real influence on medical decision-making represent an example of procedural justice.

A further issue around precision medicine concerns who decides what to invest in and how. In the UK these decisions are taken by the state, who direct health policy, and by executives who manage the NHS. The NHS is generally guided by fundamental principles of utilitarianism and described by Mill[16]as the best use of resources for the greatest good within the population.[17,18] Whilst on the surface, precision medicine using companion diagnostics appears to fit with this principle very well i.e. using expensive drugs only in those patients who are likely to benefit. However, the high cost of these medications may tip the balance in favour of non-investment in this technology in favour of cheaper preventative measures.[19]

Prescribing expensive novel oncotherapeutic agents linked to companion diagnostics will reduce therapy costs compared with providing the treatment to all patients and there is a wealth of evidence demonstrating that such agents are effective. Recent scientific developments in the molecular events of lung cancer pathogenesis mean that it is often cited as a 'role model' for precision medicine.[20] A good example is the use of tyrosine kinase inhibitors (TKIs) in the treatment of advanced adenocarcinoma of the lung.[21–25]

These agents can give overall survival benefits of 13 months and between 14 and 17

months, respectively, when used in the setting of lung cancer.23,26 It is difficult to argue against the deontological principle of a right to life27 and every patient with a terminal illness is likely to feel strong leanings towards egoism when considering their own mortality.

However, from a utilitarian perspective it may be of more benefit to more people to redirect the large sums of money involved in funding these novel oncotherapies towards early diagnosis and/or disease prevention to potentially benefit a larger number of patients.19 Many of these oncological drugs have been approved for use in the NHS by the National Institute of Health and Care Excellence. It is evident that emotive arguments from the few can influence decision-making by the state through the media and court action as was the case with Herceptin.28 The government and the media were criticised by the medical profession at the time for not being able to step back from individual terminal cancer cases and make resourcing decisions for the benefit of the many.19

Investment would be required to ensure that precision medicine services are of sufficient quality to be reliable and this would include staffing, equipment and processes that comply with clinical standards. There is a conflict that arises when balancing quality, costs and timeliness in the diagnosis of lung cancer.29 Greater efficiencies are said to occur with large batches of tests and centralisation of services in large, single centres.30–32 There is a counter argument to this approach when one considers slow turnaround times resulting from batching of diagnostic tests.33 Again, using the example of lung adenocarcinoma, it is now possible to perform EGFR receptor mutational analysis locally using a fully automated, clinically validated real-time PCR platform that yields results in under 3 h.34 A point-of-care testing service would require investment in technology and staff but has clear benefits for patients who may deteriorate in the 2–3 week wait for results from a centralised laboratories using next generation sequencing (NGS) technologies. The ToGA study35 showed that 25% of patients with gastric or gastro-oesophageal junction adenocarcinoma were denied treatment with Herceptin because they became too unwell for treatment whilst waiting for a HER-2 immunohistochemistry report which, during that study, took around two weeks to provide. Being able to offer TKI therapy also relies upon patients being well enough to receive the treatment; they need to be performance status 0 or 1.36,37 If the findings of the ToGA study are reflected in patients with disseminated lung adenocarcinoma, then there will be a significant number denied therapy that offers significant progression-free survival advantages. The number of patients who are denied such treatment is not provided in the medical literature yet and this is an area of research need for the future. This is, therefore, evidence to support investment in a hub-and-spoke model of solid tumour genetic analysis where a central hub

provides NGS back-up for localised point-of-care, rapid PCR assays that provide actionable genetic mutation results for oncologists and patients on ethical grounds of a patients' right to life and the pursuit of both beneficence and non-maleficence.9,12,18

It is possible that patients may perceive discrimination when looking at the observable characteristics of individuals receiving TKI therapy for adenocarcinoma of the lung. This is because the somatic genetic mutation within the tumour occurs with greater frequency in young, Asian females who have never smoked.38 Differences in patient smoking status may be misconstrued and perceived as bias by a treating organisation on the basis of what is perceived by society to be a harmful lifestyle for which taxpayers bear the cost.39 It may be useful to investors in this area to consider the possibility for misconception by patients. This risk may be mitigated against by the production of detailed patient information leaflets freely available in the oncology outpatient waiting room.

There is evidence of increased use of direct-to-consumer (DTC) genomic testing by patients and this also applies to sequencing of solid tumours.40 The reasons for this are unclear but cancer patients often use support groups both online and in person for emotional and social support. Whilst these networks are likely to be beneficial in psychosocial terms, they may highlight differences in oncological treatment practices across the

country.32 Autonomy, dignity and integrity and mortal desperation all drive the need to explore treatment options, including untested experimental ones. This is an example of self-interest pursuit that underpins ethical egoism and drives, in part, the DTC genetic testing market. This is at odds with utilitarian principles that form the basis of workings in the NHS. Reports generated by DTC companies have the potential to create conflict within the doctor–patient relationship where tumour molecular profiling highlights somatic genetic mutations that also have implications for the germline and hence family members.41 Healthcare professionals may encounter difficult ethical situations where maintaining patient confidentiality in the face of preventing harm to uninformed relatives becomes difficult in situations where patients refuse to discuss their medical issues with their family.41,42

Meadowcroft argues that governments have a responsibility to restrict market forces that exploit vulnerable patients who pay for tumour genomic information in the context of a terminal cancer diagnosis.35 In addition, there are problems with knowing the relevance and risk that identification of variation by genomic analysis raises and the consensus amongst medical professional is that patients should be discouraged from using these services until such time the full implication of their findings is known and evidence based.43 The counter

argument is that restricting DTC genetic testing undermines respect for patient autonomy.44

Patients are also using DTC testing for predicting future cancer risk.45 'Expert advice' received in the accompanying DTC genetic reports can be inaccurate and misleading43 and there is also evidence that people do not necessarily change their risk behaviours in accordance with the information they receive.46 These are usually healthy people who are receiving information about risk associated with single nucleotide variation linked with multifactorial risk of cancers such as colorectal cancer.47 Again, full understanding of how a person's genes interact with the environment to determine precise risk of developing such cancer is not fully understood and the provision of DTC genomic testing is not fully regulated.44,48 Some of these arguments are used to support the call for increased regulation of the DTC genetic testing industry.43,49

What may not be fully considered before taking up DTC testing in a setting of malignancy is the potential for identification of mutations that carry implications for family members through the germline.50 There are many genes that have been shown to have implications for germline inheritance that may be identified during testing for somatic mutations in

cancers,50 and the best characterised of these is BRCA1/2 associated breast and ovarian cancer syndrome.51–53 As well as dealing with the uncertainty around infrequently encountered genetic variants and knowing how they translate into phenotype, there is also the need to provide genetic counselling before testing so that patients can make an informed choice, not only for themselves but also for family members.43,54 This requirement is based on the principle of patient autonomy and informed consent. Patients (and their family members) can only make a choice based upon adequate information and influence before they proceed.15,55,56Testing and referral to genetic counselling after the event of revealing a genetic variant creates difficulties for professionals and their relationships with patients in an outpatient clinic by undermining this principle.57 Genetic testing without prior patient consent denies patients an opportunity not to know the findings.55 There is a need for investors in precision medicine to ensure there are enough genetic counsellors and clinic time to cope with the inevitable increased demand that precision medicine and genetic testing of malignant tumours will bring.49,56

In conclusion, the competing interests of utilitarianism and egoism create dilemmas for decisions regarding investment in precision medicine. The need to balance the perceive rights and needs of individuals against those of society as a whole is an on-going challenge in the distribution of limited health service resources. There is need for proper planning,

organisation and investment into precision medicine to cope with the consequences of both DTC and healthcare-directed genetic testing for genetic counselling, therapeutics and diagnostic networks. Consideration needs to be given to providing adequate time and training to allow for meaningful shared decision-making with patients and there is a strong case in support of a hub-spoke model to provide rapid, solid tumour genetic mutational analysis to prevent patients missing out on beneficial treatments.

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Appendix B

Integration of rapid PCR testing as an adjunct to NGS in diagnostic pathology services within the UK: evidence from a case series of non-squamous, non-small cell lung cancer (NSCLC) patients with follow-up.

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Abstract

Aims Somatic genetic testing in non-squamous, non-small cell lung carcinoma (NSCLC) patients is required to highlight subgroups eligible for a number of novel oncological therapies. This study aims to determine whether turnaround times for reporting epidermal growth factor receptors (EGFR) by next-generation sequencing (NGS) alone is sufficient to meet the needs of lung cancer patients.

Methods We performed a retrospective case series with follow-up. Outcomes of EGFR testing (102 tests) in 96 patients by NGS were compared with a rapid, fully automated PCR-based platform (Idylla) in local histopathology laboratories.

Results Turnaround time for reporting NGS was 17 calendar days. Reporting using the Idylla EGFR Mutation Test, by contrast, gave a potential turnaround time of

3.8 days from request to authorisation. Three-quarters of patients presenting with stage IV disease had a performance status of 0, 1, or 2 but 18% experienced rapid clinical deterioration ($p<0.05$). A third of these patients were deceased by the time NGS reports were available.

Conclusions We discuss issues around integrating rapid PCR testing alongside NGS in multidisciplinary care pathways and strategies for mitigating against foreseeable difficulties. Dual testing for stage IV non-squamous, NSCLC patients has the potential to improve care and survival outcomes by providing access to the right test at the right time.

Introduction

Lung cancer is responsible for the highest mortality rate for all cancers across the globe and the UK has the poorest survival outcomes of developed nations.[1] Somatic genetic testing in non-squamous, non-small cell lung carcinoma (NSCLC) patients is required to highlight subgroups eligible for a number of novel oncological therapies.[2] Epidermal growth factor receptors (EGFRs) are membrane bound tyrosine kinase receptors that form part of the ErbB transmembrane growth factor family of receptors found on in a wide range of human tissues including lung and bronchus.[3] Somatic EGFR mutations in non-squamous, NSCLC are key mutational drivers of malignant behaviour through downstream alterations in downstream cell signalling pathways such as mitogen activated protein kinase (MAPK).[4 5]

In Caucasian populations the occurrence of EGFR mutations in lung adenocarcinoma is in the region of 15% and tends to be more frequently, but not exclusively, seen in female, never- smokers.[6] Up to 90% of activating EGFR mutations occur as a missense point substitution in exon 21 resulting in an arginine amino acid residue at position 858 of the protein instead of the normal leucine (L858R) or as a small in-frame deletion of nucleotides in exon 19.[7–10]

The Idylla platform (Biocartis, Belgium) is a fully automated system based on PCR technology that can detect 51 activating mutations in formalin-fixed paraffin embedded (FFPE) tissue samples in exons 18 through 21, including G719/A/CS, L861Q, S768I, L858R, insertions in exon 20 and multiple deletions in exon 19 plus the most common resistance mutation, T790M.[11–17] Arguably, the most valuable attribute of this system is to provide these findings within histopathology departments near to oncology patients as results can be achieved in as little as 3 hours, and this has been reported widely as a major benefit to patients with late-stage disease.[18–21] Given that lung cancer accounts for the most deaths due to cancer world-wide, there is an opportunity to make a great impact on patient outcomes with earlier intervention.[1 22 23]

Tyrosine kinase inhibitor (TKI) drugs directed at EGFR mutations in non-squamous NSCLC are considered highly effective at extending progression-free, and overall survival in patients with lung cancer.[8 24 25] Development of third-generation TKIs also provide treatment options for patients on TKI therapy who relapse due to the development of resistance mutations in EGFR.[26]Osimertinib, for example, has become a first-line treatment option for patients with EGFR mutant lung cancer during the COVID-19 pandemic.[27 28]

Integrating molecular diagnostics within histopathology laboratories also offers the opportunity to optimise the preanalytical preservation of tissue to maximise the quality of mutational analysis outputs while also maximising the use of limited tissue samples. Less

FFPE biopsy tissue (5 µm) is required for the Idylla EGFR Mutation test as compared with DNA next generation sequencing (NGS) which uses 60 µm.[29 30] The Idylla system is said to generate valid results with tumour neoplastic cell content as low as 10% in tissue sections and needs minimal time and human resource inputs with no batching requirements to maintain economic viability.[12 14 18 31 32] Fully integrating solid tumour genomic findings with the phenotypic, cellular pathology data is essential for meaningful and safe clinical interpretation by pathologists and oncologists.[29 33]

This is a service development study that looks to assess turnaround times for reporting both NGS and rapid PCR testing of EGFR mutations in eligible patients with lung cancer to identify if there is justification for service improvement. We go on to find which patients may benefit and discuss options for integrating PCR methods alongside our current testing method based on NGS. NGS is performed at an external laboratory to the cellular pathology department and so requires sending 60 micrometres of FFPE tissue on glass slides by post. To our knowledge, this is the largest concordance study in the UK to assess somatic EGFR mutational testing by Idylla compared with NGS to date.

Methods

Case selection

Consecutive lung cancer patient samples with a histopathological diagnosis of non-squamous, NSCLC were tested for EGFR mutation by DNA panel NGS as per routine reflex requesting by cellular pathologists from 1 January 2020 onwards were included for prospective comparisons with findings using Idylla rapid analysis. The minimum tissue requirement was 100 cells to proceed with companion diagnostic testing. EGFR PCR testing by Idylla was performed on remaining archival FFPE tissue after completion of the existing diagnostic pathway and authorisation of the pathology report as agreed by the Joint Study Review Committee who designated this study service development. Patient samples were pseudoanonymised and recorded by accession number.

Case mix

All patient sample types were used in analysis including lung resection specimens, image- guided core biopsies, bronchial biopsies, endobronchial ultrasound-guided (EBUS) fine- needle aspiration cytology (FNAC) specimens and diagnostic cytology specimens, for example, pleural effusion.

Preanalytical considerations

Histological tissue samples were fixed in 10% neutral buffered formalin for between 6 and 72 hours as required to produce clinically valid programmed death ligand 1 (PD-L1)

immunohistochemistry (IHC) tumour proportion scores. EBUS samples in our institution are placed in 10% neutral buffered formalin and processed as small tissue samples by short cycle processing.[34] Non-gynaecological diagnostic cytology samples such as needle aspirates and washings are preserved in Cytolytsolution containing methanol for fixation where appropriate. Serous effusions are received in a sterile universal container and refrigerated until processing. Excess fluid, after preparation of a Giemsa and a Thinprep Papanicalou- stained slide for diagnostic purposes, is kept in cold storage at 4°C until a cell block is requested by a pathologist. Cell block FFPE sections were used in the Idyllasystem for serous effusion fluids. Cell blocks were prepared using the bovine thrombin and fibrinogen technique. Two negative control cell blocks free of human cells were used as part of the system verification to ensure no interference of bovine genomic contaminants.

Next generation sequencing

DNA panel testing by NGS for EGFR, KRAS, NRAS, PIK3CA, CDKN2A, PTEN, RET, BRAF and ERBB2 (HER2) was carried out using Roche pan cancer panel (custom-design) at an external laboratory using a total of 60 µm of FFPE tissue in 10 sections on air-dried unstained glass slides. A minimum of 50 ng of DNA is required from the tumour FFPE sections for analysis and sections should contain at least 10% malignant nuclear content.

Idylla EGFR mutation test

Study participants were blinded to NGS results prior to undertaking the Idylla EGFR mutation test. One 5 µm thick tissue roll was cut from the FFPE tissue block using a fresh, clean microtome blade on a designated molecular microtome. Water baths were not used in specimen preparation to avoid contamination. Following completion of the usual diagnostic pathway a subsequent H&E slide was examined and assessed for percentage tumour nuclear content (TNC) as a consensus score between two independent observers (AF and TJ) and tumour area was also recorded as a separate variable according to manufacturer instructions (GD). The next sequential slice was used in the Idylla EGFR mutation test cartridge to ensure accuracy of TNC in analysis. Macrodissection was performed on the cutting plate of the molecular microtome to achieve minimum 10% TNC were appropriate. The FFPE tissue roll was sandwiched between two discs of filter paper moistened with sterile water to prevent movement of the tissue section in the Idylla EGFR Mutation Test cartridge as per manufacturer guidelines. Hands on preparation time by biomedical staff was 5 min.[31]The Idylla platform autoextracts DNA for analysis inside the closed and sterile cartridge to prevent contamination. The presence of mutation specific DNA is detected by fluorescence- based multiplex qPCR using PCR reagents present in the cartridge which is then recorded by the

console. A CQ value is given for each fluoresent amplification curve and a lower value is an indication of the presence of more target DNA. A CQ value of 20 equates to 200 ng of DNA, as determined by the manufacturer when determining the limit of detection of the test. Upper limit of clinical validity for a no mutation detected result was defined as a CQ of 26 or more. A positive result by the Idylla EGFR Mutation test represented detection of an actionable EGFR mutation. The CE-IVD vaIidated Idylla EGFR Mutation Test uses FFPE tissue sections from human NSCLC tissue. In this study, all NSCLC samples were used including cytological FFPE samples.

Statistical analysis

Statistical analysis was performed using SPSS V.26.0.0 statistical package from IBM for descriptive statistics. Fisher's exact test was used to assess for differences in categorical variables using 0.05 as the threshold for statistical significance. 95% CIs for proportional data were calculated using the formula CI=p± z × square root of p (1-p)/n where p is sample proportion and n is the sample size with a z score of 1.960.

Results

Two bovine thrombin-fibrinogen cell free paraffin blocks were processed by standard protocols and used as negative cytology controls for the Idylla EGFR mutation test. Both these tests returned an invalid result reflecting absence of a DNA template for analysis.

We prospectively compared the EGFR mutational status of 96 lung adenocarcinoma patients who had DNA panel NGS testing requested either by members of the lung cancer multidisciplinary team (MDT) or by the pathologist as part of reflex requesting between 17December 2019 and 28 October 2020. Exhaustion of Idylla EGFR mutation test cartridges defined the study close out.

Of the total number of patients tested, 47 were male (49%) and 49 were female (51%). The mean age of patients in our sample overall was 72 (range age 41–92). The mean age of patients in the clinical stable group (n=79) was 75 years (range 41–92) and in the smaller group who deteriorated rapidly (n-17), the mean age was 63 (range 57–83).

EGFR requests

There were 102 Idylla EGFR mutation tests performed for 96 patients; 6 patients had repeat testing performed on different specimens or an alternative block where DNA yield was insufficient. Two patients (2.1%) had two tumours in a resection specimen and both were analysed for EGFR; One patient had repeat Idylla testing following an invalid result due to

insufficient cellular material and three patients NGS failed due to poor quality or insufficient DNA input.

Analysis of the number of cases by month reflected a change in requesting patterns during the (SARS-CoV-2) pandemic (see online supplemental file). There was a reduction in the number of non-squamous, NSCLC diagnosis during this time as a compound effect of a number of variables including reduced biopsy appointment slots and a reduced number of patients presenting to their GP or emergency department.

Fourteen (14.58%) patients had an EGFR mutation and eleven of these (78.57%) were either exon 19 deletions or the L858R mutation (see figure 1 for details). These figures are in keeping with expected incidence of EGFR mutations in the UK.[6–8 35]

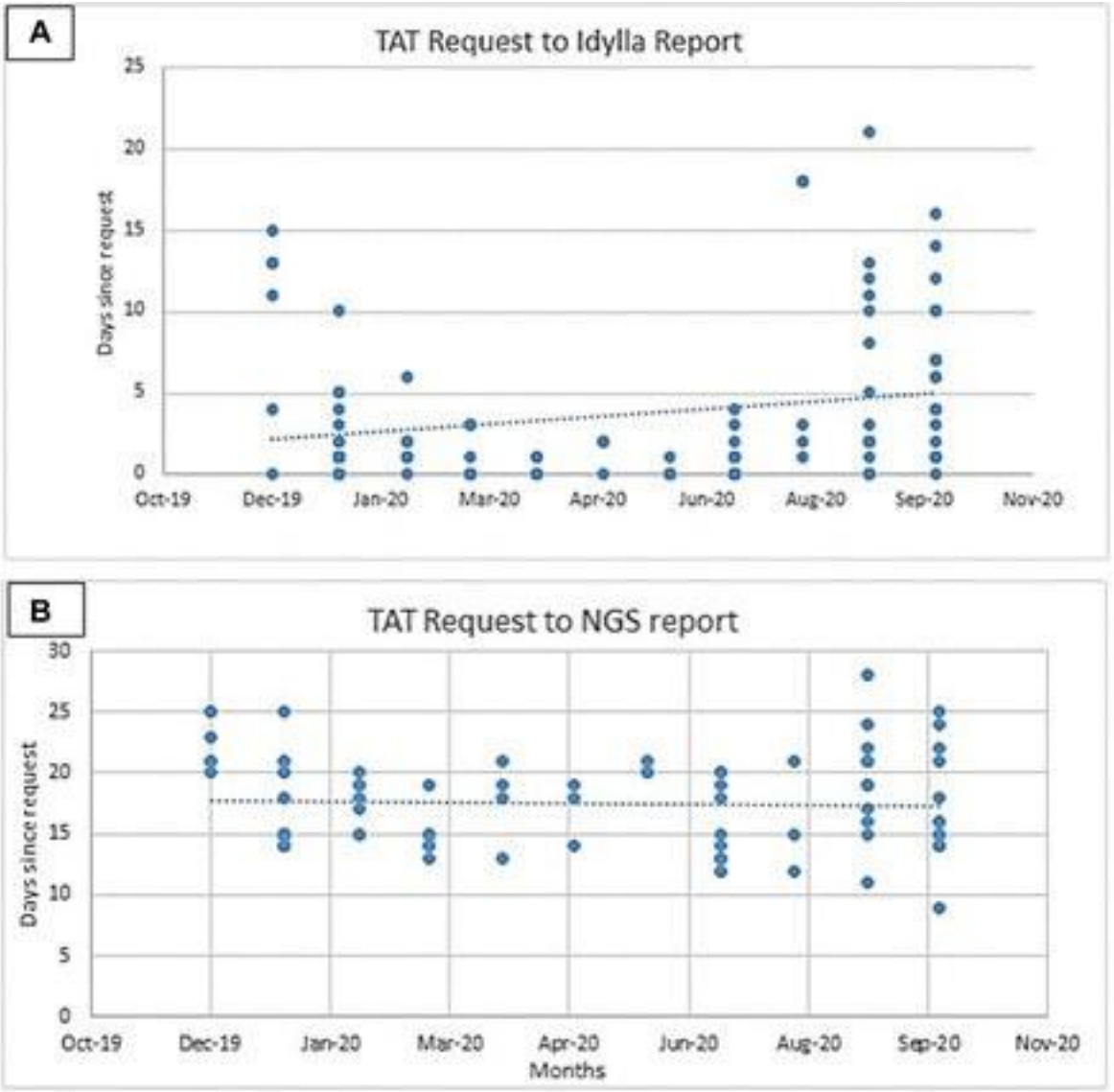

Figure 1

Turnaround time (TAT) for reporting EGFR by different methods. (A) TAT to

report EGFR by rapid PCR method in local histopathology laboratory. Mean 3.8 days. (B) TAT for reporting of next generation sequencing (NGS) by external laboratory. Mean 17 days. EGFR, epidermal growth factor receptors.

A total of 31 patients (32%) (95% CI 22.7% to 41.3%) were deceased by conclusion of the study (10 months). Seventeen patients (17.7%) (95% CI 10.1% to 25.3%) were identified as having deteriorated rapidly according to the medical notes (See table 1). We defined this as a reduction in two or more Eastern Cooperative Oncology Group (ECOG) performance status (PS) scores within 8 weeks of histological diagnosis. Six per cent of the patient group were dead before the NGS report was available. Of the 17 patients who deteriorated rapidly, three were identified as having an actionable variant in EGFR that could have been treated with TKIs. One of these patients had brain metastases at presentation with a PS of 1 and died a week before the NGS was reported as showing an exon19 deletion. The turnaround time for this particular patient's NGS report was 15 calendar days. The other two patients with actionable variants deteriorated to PS4 before the NGS report was available and became ineligible for TKI therapy. Both patients died shortly after the NGS reports which was turned around in 18 calendar days (L861Q mutation) and 28 calendar days (L858R) (see figure 1 for turnaround time calculations). Mean turnaround time overall for DNA NGS incorporation into histology report and authorisation was 23.3 days from request to report being emailed to pathologist and oncologist. The turnaround time of 23.3 days does not include time for laboratory staff to cut sections, track on our laboratory information system and package for posting to an external laboratory. Mean time to NGS panel report emailed to the oncologist was 17 days from the day of slides were sent from our laboratory. Time taken to report the EGFR result by rapid PCR using the Idylla platform was 3.8 calendar days. This is a real-world reflection of time for the EGFR testing request to be acted on by laboratory staff required to cut the sections for cartridge input and includes estimated time for pathologist checking and authorizing.

Table I

Specimen types, diagnoses and stage of disease

- Red indicates stage IV patients with distant metastatic disease; green indicates good to moderate PS.102
- EBUS,endobronchial ultrasound-guided cytology; ECOG, Eastern Cooperative Oncology Group; FNAC, fine-needle aspiration cytology; NOS, not otherwise specified; NSCLC, non-small cell Jung cancer; PS, performance status; SCC, squamous carcinoma.

Clinicopathological features	Total (n=96)	Clinically stable patients (n=79)	Patients who rapidly deteriorated (n=17)
Age (years), mean, (range)	72 (41–92)	75 (41–92)	63 (57–83)
Sex p=0.187			
Female	49	43	6
Male	47	36	11
ECOG PS at histological diagnosis p=0.215			
PS0	17	13	4
PS1	41	37	4
PS2	28	24	4
PS3	8	5	3
PS4	0	0	0
Not recorded	1	0	1
Stage at histological diagnosis p=0.049			
I	11	10	1
II	5	5	0
III	17	16	1
IV	47	33	14
Metastatic recurrence	16	15	1
Specimen Type, n (%) p=0.534			
Lung resection	7	7	0
Lung core biopsy	36	30	6
Bronchial biopsy	5	4	1
Pleural biopsy	2	1	1
Lymph node core biopsy	14	9	5
Skin or soft tissue biopsy	6	6	0
Bone	3	3	0
Pleural fluid	4	3	1
EBUS	16	13	3
FNAC lymph node	1	1	0
Other (pericardial fluid, liver and breast)	2	2	0
Smoking status, n (%) p=0.115			
Never smoked	7	4	3
Ex-smoker	46	41	6
Ex-smoker, now vaping	3	2	1
Current smoker	32	29	3
No recorded	8	4	4
Histological diagnosis, n (%) p=0.169			
Adenocarcinoma, poorly differentiated	48	40	8
Adenocarcinoma, moderately differentiated	24	18	6
Adenocarcinoma, well differentiated	8	8	0
Adenocarcinoma mucinous or enteric-type differentiation	5	2	3
Adenosquamous	2	2	0
Large cell neuroendocrine carcinoma	1	1	0
NSCLC, NOS (non-SCC/ p40 negative)	2	2	0

Table 2

Treatment decisions and outcomes for patients with EGFR mutations identified in this case series

Sex	Smoking status	Stage	PS	Mutation by DNA NGS	Treatment	Outcome	Comments
F	Never	IVB	1	Exon 19 del	Gefitinib commenced February 2020. Single agent pembroluzimab commenced December 2020 (PD-L1 95%)	Radiological progression July 2020 (no T790M mutation). Alive at f/up January 2021.	Insufficient tissue left for Idylla in diagnostic biopsy. Exon 19 deletion detected in bone biopsy October 2020
M	NR	IVB	1	Exon 19 del	BSC	Died before NGS reported.	PS1-5 within 2 weeks. Exon 19 deletion detected by Idylla while PS1
M	40 /day	IIB	1	L816Q	ITU for hospital acquired pneumonia post-CT guided lung biopsy	Died 9 days after NGS report.	PS1-4 within 1 week.
F	Ex	IVB	2	Exon19 de	Gefitinib	Died 7 months after starting TKI	Delay starting Gefitinib of 2 months due to orthopaedic surgery for pathological hip fracture.
F	Never	IB	1	L858R	Stereotactic radiotherapy. Disease stable on CT.	Alive at last f/up December 2020	
M	Ex	IVA	1	Exon 19 del	Afatinib commenced June 2020	Alive at last f/up December 2020	Mild side effects skin rash and grade one diarrhoea.
M	Ex	MR	0	G719C and S768I	Unknown (treated in a different health board)	Alive at last follow-up	Previous lobectomy for T1c N0 adenocarcinoma 2019.
M	Ex	IVB	1	Exon 20 ins	BSC	Died 7 weeks after NGS report	PS1 –3 in 5 weeks.
F	Ex	IVA	1	L858R	First line osimertinib (COVID-19 guidelines)	Alive at last f/up December 2020	Side effects grade one diarrhoea and mouth ulcers
F	Ex	IVA	1	Exon19 Del	Treatment with combined pembroluzimab and pemetrexed/carboplatin	NGS report 24 days after PD-L1, ALK-1 and ROS-1 IHC reported. Alive last f/up December 2020	Chemotherapy/immunotherapy started before EGFR result known with patient fully informed. Continued as good radiological response.
F	Ex	IVB	1	Insufficient DNA for NGS	BSC	Rapid deterioration. Unable to have repeat biopsy. Died from PE	L858R substitution detected on Idylla Tumour nuclear content 50%
M	Ex	IVA	1	Exon 19 del	Afatinib commenced Aug 2019. Stopped Sept 2020 as radiological progression.	Died November 2020	T790M not detected NGS or Idylla on disease progression.
M	Never	IVA	2	Exon 19 del	Afatinib commenced October 2020.	Alive at last f/up December 2020	East Asian heritage. Side effect of skin rash.
F	NR	IVA	2	NGS request cancelled: patient deterioration	BSC	Alive at last f/up by palliative care December 2020	PS2- PS3 in 1 week. Idylla detected L858R

- ALK-1, anaplastic lymphoma kinase-1; BSC, Best Supportive Care; EGFR, epidermal growth factor receptors; IHC, immunohistochemistry; ITU, Intensive Therapy Unit; NGS, next-generation sequencing; NR, not reported; PD-L1, programmed death ligand 1; PE, Pulmonary Embolus; PS, performance status; ROS-1, ROS proto-oncogene 1; TKI, tyrosine kinase inhibitor.

Table 2

Treatment decisions and outcomes for patients with EGFR mutations identified in this case series.

Clinical impact

Understanding that 6% (95% CI 1.2% to 10.8%) of our patient case series were dead before the NGS report was available prompted a retrospective analysis of clinical data in an attempt to try to identify which subsets of patients were at risk of rapid clinical deterioration. We also sought to identify if there was a window of opportunity to make a difference to survival outcomes by rapid somatic tumour testing for EGFR. The only clinical parameter associated with rapid deterioration was the stage of disease at time of histological diagnosis. Stage IV patients were at risk of rapid clinical deterioration ($p<0.05$) and three quarters of this group had a PS of 0, 1 or 2 at time of MDT discussion (tables 1 and 2).

Test performance

The service development project was designed to only test patients for EGFR after

completion of current standard of care in the histopathology laboratory. Of 102 tests performed, agreement between the two test modalities was achieved in 96.39% (95% CI 92.8% to 100%) of test events where there was sufficient tissue for testing (table 3). There were 11 (10%) (95% CI 4.2% to 15.8%) occasions where NGS failed to extract sufficient DNA from a test sample of 60 µm of tissue and Idylla was able to produce a valid report in nine of those instances using 5 µm of tissue remaining in the FFPE block. Of these 11 cases, two were cytology samples (one EBUS and one lymph node FNAC washings) and the remainder were tissue biopsies. Of these tissue biopsy samples that failed there was one bone biopsy, one bronchial biopsy, and three lymph node core biopsies with the remainder being CT-guided lung core biopsies. Two of the nine tests that failed NGS included patients where an L858R mutation was detected by Idylla that went undetected by NGS; both of these samples were lymph node core biopsies. In one case, NGS was able to report an exon19 deletion that went undetected by Idylla due to insufficient residual tissue for testing by Idylla. Interestingly, this patient required further EGFR analysis 9 months later following disease progression to test for presence of resistance mutations. On that occasion, with sufficient tissue being available for both NGS, followed by Idylla, there was agreement regarding the presence of an exon19 deletion in the absence of a T790M resistance mutation. Both the NGS and Idylla failed to produce a valid report for two CT-guided lung core biopsy specimens (1.9%) due to insufficient remaining FFPE tissue.

Table 3

Concordance analysis of Idylla with next-generation sequencing (NGS) for EGFR mutations in lung non-squamous, non-small cell carcinoma.*

	NGS	Del Ex19	Ins Ex20	L858R	L861Q	G719X	G719x, S768I	T790M	S768I	Other	No mutation	Insufficient or cancelled	Total
Idylla													
Del Ex19		7											7
Ins Ex20			1										1
L858R				2								2	4
L861Q					1								1
G719X						0							0
G719X, S768I							1						1
T790M								0					0
S768I									0				0
Other										0			0
No mutation											71	7	78
Insufficient		1									7	2	10
Total		8	1	2	1	0	1	0	0	0	78	11	102

- *Green indicates concordant results. Red indicates discordant results.
- EGFR, epidermal growth factor receptors.

Table 3

*Concordance analysis of Idylla with next-generation sequencing (NGS) for EGFR mutations in lung non-squamous, non-small cell carcinoma.**

Analysis of the clinical record revealed variation and complexity in treatment decision making in patients with lung cancer with advanced disease. One patient within the group identified as having an EGFR mutation was commenced on combined chemotherapy and checkpoint inhibitor while waiting for NGS DNA panel results to become available.

Treatment decisions may also be affected by other considerations such as recent surgery. Eleven of the 14 (78.5%) patients in this group were stage IV with an additional one having metastatic recurrence a year after surgical resection.

Discussion

Turnaround time for reporting: opportunities for intervention

Timeliness of reporting genetic mutations in solid malignancies is important for ensuring that patients get the most appropriate treatment available at a time when they are sufficiently well enough to tolerate side effects and achieve a progression free survival advantage. Real- world data regarding biomarker testing in patients with lung cancer is limited. This study provides evidence that in real-world practice some patients are commenced on standard chemotherapy plus/minus checkpoint inhibitors while waiting for NGS DNA panel reports to be completed. A study by Ruggiero *et al* found a similar phenomenon with only 79% of EGFR positive advanced lung cancer patients in the US commenced on appropriate TKI therapy.[36] Similar data has recently been published regarding advanced lung cancer patients in England with 71% of patients receiving TKI therapy on identification of an EGFR mutation.[37] It was not clear from their paper which analysis method was used to detect EGFR mutation and they restricted their analysis to patients with a PS of 0, 1 and 2.[37] The authors conclude that it is the timeliness of reporting EGFR mutation, in addition to anaplastic lymphoma kinase-1 (ALK-1) and PD-L1 status that is responsible for patients not receiving therapy for which they are eligible.[37] Furthermore, it was demonstrated in Germany that up to 20% of EGFR mutation positive patients start chemotherapy instead of TKI therapy because they are clinically unable to wait for NGS results to be made available.[38] This therapeutic window has been shown to be small in other cancer types also. The ToGA trial of herceptin treatment in HER-2 positive advanced gastric adenocarcinomas found that as many as 25% became ineligible for treatment while

waiting up to 2 weeks for IHC to be reported.[39]Our study findings are in keeping with these studies. We illustrate that 18% of patients deteriorate rapidly and one-third of these patients were deceased before NGS results were available.

Hardtstock *et al* show that increasing age, no systemic therapy, stage IV disease and chronic comorbidities correlated with a poorer overall survival for patients with lung

cancer.[38] However, rather than looking at survival data, we tried to identify clinical parameters that highlight lung cancer patient subgroups who could benefit from rapid EGFR testing. This study indicates that those most likely to deteriorate are stage IV patients and that they appear well enough for TKI therapy at point of first MDT discussion. There is a clear window of opportunity to improve on current practice. This has recently been acknowledged by National Health Service (NHS) England guidance that describes a salvage testing pathway for sick patients with advanced lung cancer who would not survive to see the potential beneficial repercussions of NGS-based mutation detection.[40] While they do not endorse a specific testing modality, it allows local testing to continue by rapid PCR methods in a context of genomic testing in centralised laboratory hubs.[40]

Optimal use of limited tissue samples in a context of a requirement for multiple predictive biomarkers

Judicious use of limited tissue samples to obtain the most clinically relevant information for patients has been the remit of pathologists for many years. Recommendations are for the use of only two diagnostic IHC stains (p40 and TTF-1) for poorly differentiated cases of NSCLC to help determine cell lineage. This recommendation is borne out of the need to preserve tissue for additional predictive biomarkers in relevant patients. It Is also advisable to precut tissue sections in the laboratory before the pathologist makes a diagnosis as every time the FFPE tissue block is re-cut for additional slides a small amount of tissue is lost in alignment.[35 41] Optimal use of tissue will help to prevent the need for re-biopsy of patients which, in the case of CT-guided lung biopsy, for example can come with potentially life-threatening complication of haemoptysis and pneumothorax.[42] It is also imperative that pathologists be empowered to make choices regarding the most appropriate method for predictive biomarker testing based on the material in front of them through ‘reflex

standard tissue processing in the histopathology laboratory mandates 6–72 hours of fixation in 10% neutral buffered formalin for clinically actionable PD-L1 interpretation and also for diagnostic IHC.[35 46]

It is not possible to consider EGFR testing in isolation when considering the ongoing treatment options for patients with lung cancer. The pace of change in molecular testing in

lung cancer is rapid.[43] Multidisciplinary practice in diagnosis, predictive marker testing and treatment needs to be sufficiently well resourced to allow and promote flexibility in healthcare. Current guidelines from the European Society for Medical Oncology recommends molecular testing for established predictive biomarkers EGFR, ALK-1, ROS-1 BRAF, NTRK and PD-L1. PD-L1 assessment currently relies on an IHC assay of protein expression rather requesting'.[37] There are myriad options available to pathologists for some markers, such as ALK-1 which include IHC, fluorescence in-situ hybridisation, PCR and RNA sequencing by NGS.[37 43]Sometimes the best choice will be the one that consumes the least amount of tissue. A robust clinical diagnostic laboratory will have unhindered access to all options in our view. It can be demonstrated that using a combination of IHC and Idylla can limit consumption of tissue to 26 µm (IHC 16 µm plus 10 µm for Idylla) (see table 4). However, nearly five times more tissue is consumed to meet current requesting requirements if one chooses to prioritise NGS methods (124 µm). The RNA sequencing panel available in Wales will also yield information regarding Met exon14 skipping lesions and Ret fusions. These additional genes are also analysed in the single cartridge Idylla Gene Fusion Assay which takes around 3 hours to yield results.[44]

Table 4

Tissue consumption in predictive biomarker testing

	Technique			
Predictive Biomarker	IHC	FISH	NGS	Idylla
EGFR	NR	NA	60 (+KRAS)	5
BRAF	NR	NA		5
ALK-1	4	8	50 (+MET and RET)	5 (+MET and RET)
ROS-1	4	8		
NTRK 1,2,3	4	24		
PD-L1	4	NA	NA	NA

- Tissue consumption is measured in micrometres (µm).

ALK-1, anaplastic lymphoma kinase-1; BRAF, B-Raf proto oncogene; EGFR, epidermal growth factor receptor; FISH, fluorescence in situ hybridisation; IHC, immunohistochemistry; KRAS, KRAS proto-oncogene; MET, MET proto-oncogene; NA, not available; NGS, next-generation sequencing; NR, not recommended; NRTK, neurotrophic receptor tyrosine kinase; PD-L1, programmed death ligand 1; RET, RET proto-oncogene; ROS-1, ROS proto-oncogene 1.

Proposed biomarker testing algorithm

We suggest that identification of stage IV patients be made on histopathological request forms at the time of biopsy to allow for appropriate reflex requesting of molecular tests at the point of microscopic diagnosis. Reflex requesting is recommended in the UK and refers to pathologist initiated testing rather than waiting for discussion at MDT to request predictive biomarkers in an effort to minimise delays in results for therapeutic decision making.[43] The suggested biomarker requesting algorithm assumes that there is sufficient tumour malignancy for testing, preferably with low cold ischaemic time and appropriate fixation. Fixation may vary depending on institution and sample type. For example, use of serous fluid cytology for testing may yield good quality and quantity of DNA and RNA fragments in situations of minimal exposure to 10% neutral buffered formalin or fixation in methanol.

Molecular diagnostic services need also to be prepared to test for KRAS,[47] MET skipping lesions,[48 49] RET fusions[50 51] and HER2[52] in the near future.[43] Our current regional testing options prioritise NGS panel testing using RNA and DNA separately to look for structural rearrangements in ALK-1, ROS-1, RET, NTRK1,2,3, MET and RET in addition to the DNA panel for EGFR, BRAF and KRAS respectively.

See table 4 for tissue consumption comparisons. In some institutions, large sequencing panels that analyse hundreds of genes are used alongside Idylla to balance rapid clinical decision making with access to experimental therapies.[53] This may represent the best compromise for maximum benefit of Stage IV non-squamous, NSCLC patients (see figure 2).

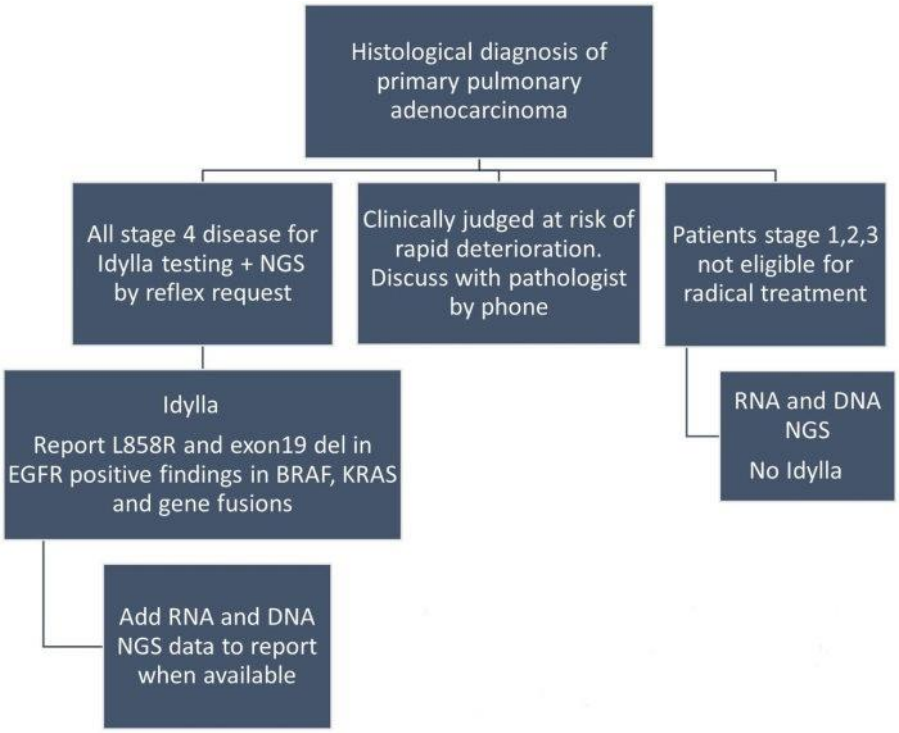

Figure 2

Proposed algorithm for predictive molecular testing in non-squamous, NSCLC. BRAF, B-raf proto-oncogene; DNA, deoxyribonucleic acid; EGFR, epidermal growth factor receptors; KRAS, KRAS proto-oncogene; NGS, next-generation sequencing; NSCLC, non-small cell lung carcinoma; RNA, ribonucleic acid.

One potential problem of integrating rapid PCR findings alongside NGS findings from the same tissue block is the potential to produce conflicting results and clinical confusion. For example, if the Idylla EGFR Mutation Test cartridge generated a negative result based on the 51 primer sets it contained there still remains the possibility of finding a novel variant in any downstream NGS sequencing carried out. This conflict could be mitigated against by only reporting positive findings from the rapid PCR test. This would allow patient benefits to be realised without oncologists having to account for any discrepancy in test results nor require patients to come back to clinic for a change in therapy. Furthermore, to simplify interpretative reporting for pathologists one could also consider restriction to reporting only exon 19 deletions or L858R substitutions which, combined, make up 90% of known actionable mutations in EGFR.[10 54 55] It should be noted that not all novel variants detected by NGS will have known clinical consequences or allied treatments. If the prioritisation of NGS for mutation detection is for gathering new information about variants of unknown significance then this runs the risk of appearing to be unconsented research not reviewed by an NHS

ethics committee. Furthermore, public bodies need to be seen to be investing public monies wisely and there is a question as to whether use of extensive NGS gene panel testing merely subsidises the pharmaceutical industry to recruit patients for clinical trials.

Feasibility of use of rapid PCR in the histopathology laboratory

The Idylla EGFR Mutation Test is a feasible choice for our histopathology laboratory because of the minimum staffing time required for preparation and reporting biomarkers without the need for batching cases. Additional molecular grade clean space for nucleic acid extraction is also not required. The Idylla testing platform allows all of the current recommended predictive molecular biomarkers to be tested with the exception of PD-L1 which relies on a morphological assessment of an immunohistochemical stain in a tissue section. Advantages of the Idylla platform include the ability to report biomarkers on the same day of request, potentially while the patient waits in the outpatient department in urgent cases.[14 56] In a context of limited tissue, Idylla has the benefit of being able to detect actionable mutations where there is insufficient DNA and RNA for NGS as supported by our data. We found that in 10% of our cases, Idylla could still detect actionable mutations where NGS failed even after 60 µm of tumour tissue has been consumed prior to the Idylla EGFR mutation test.

Limitations

Small numbers of patients limit the generalisability of the findings as a consequence of resource limitations. However, prospective collection of concordance data from one centre with one external provider of NGS reduces testing variability. The study design was for additional testing by Idylla only completion of testing by current agreed standards to mitigate any impact on patients. There were seven cases where there was insufficient tissue remaining for testing by Idylla even though the platform only requires a single 5 µm FFPE tissue section. This hampers the ability to construct a comparative analysis with a fair chance of both systems performing to the best of their ability.

Conclusions

This service development project highlights the turnaround time differences for in-house reporting of automated, rapid PCR-based EGFR analysis in comparison with NGS by an external laboratory. The difference could allow for a drastically shortened report of actionable common variants in EGFR which make up 90% of known mutants that can respond to TKI therapy.[10 54] This study shows that patients presenting with distant metastases (stage IV disease) are more likely to experience rapid clinical deterioration (18%) and that three-quarters of this group have a PS of between 0 and 2 inclusive at MDT discussion. A small (6%) proportion of the patient sample (but one-third of the patients who deteriorated rapidly) were

deceased before the NGS report of EGFR, BRAF and KRAS was available.

This represents a significant opportunity for intervention and potentially improved cancer survival outcomes. To our knowledge, this is the first study based on British data to describe the proportion of patients with lung cancer with non-squamous, NSCLC who become ineligible for EGFR-based TKI therapy due to clinical deterioration while waiting for NGS test results. Strategies for implementing rapid, automated PCR testing in local histopathology laboratories alongside existing NGS services should consider ways to mitigate the prospect of conflicting findings by two separate methods for the benefit of both patient and oncologist.

Pathologists may choose to restrict themselves to reporting of positive PCR results only to address this issue. Deference to later NGS findings, where rapid PCR is negative, will allow for determination of the clinical relevance of any rare of novel variants detected in NGS and potential eligibility for entrance into clinical trials, that is in cases where patients survive long enough to have the opportunity for such collaborative decision making.

Take home messages

- Eighteen per cent of stage 4 NSCLC patients with non-squamous, NSCLC histology are at risk of rapid clinical deterioration and ¾ of these have a performance status 0, 1 or 2 at first MDT discussion.
- Idylla EGFR mutation test—NGS concordance was 96.39% (95% CI 92.8% to 100%) based on results from 102 tests.
- Turnaround times for reporting somatic mutations in EGFR could be improved by using rapid, automated PCR-based testing that can yield results in as little as 3 hours.
- Rapid PCR testing on the Idylla platform could be integrated alongside NGS for optimum patient management. Consideration should be given to the wider genomic testing context and whether to extend rapid testing to cover other actionable variants and fusions in lung cancer.

Data availability statement

Data are available on reasonable request. Please contact first author by email with reasonable data requests.

Ethics statements

Patient consent for publication

Not applicable.

Ethics approval

Swansea Bay University Health Board Joint Study Review Committee deemed the study proposal as a service development project without need for NHS ethical approval.

Acknowledgments

Thanks to Mrs Kate Murphy at Swansea Bay University Health Board for training G Davies in using the Idylla instrument.

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Appendix C

Improving care of melanoma patients through efficient, integrated cellular-molecular pathology workflows using tissue samples with low tumour nuclear content.

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Abstract

Aims The aim of this quality improvement project was to improve the turnaround time of B-raf proto-oncogene (BRAF) mutation testing in patients with malignant melanoma to support oncologists in making timely treatment decisions.

Methods This is a prospective in-house verification of the Idylla BRAF test as compared with DNA panel next-generation sequencing (NGS) performed at an external laboratory.

Results The Idylla BRAF test had an overall concordance of 95% compared with NGS. This was considered sufficiently good for use in patients with a poor performance status who were at risk of rapid clinical deterioration. Reliable results can be generated using the Idylla BRAF test in tissue sections with tumour neoplastic cell content below 50%. We present a multidisciplinary clinical care algorithm to support dual testing.

Conclusions The Idylla BRAF test has the potential to make a significant positive impact on progression-free survival of malignant melanoma patients due to its rapid turnaround time. The Idylla BRAF test can be used as an adjunct to NGS for timely management of patients, particularly those with a poor performance status at presentation.

Introduction

Malignant melanoma is a common non-epithelial malignancy of the skin that was frequently fatal before advances in precision medicine. Treatment of malignant melanoma of skin with nodal metastases and/or distant metastases has two options for the clinical oncologist. One can use immune checkpoint inhibitors that target either programmed death ligand (PD-1) or anti-cytotoxic T-lymphocyte associated protein 4 (CTLA-4) agents or a combination of both to promote the host immune system to defeat the malignancy.[1–5] The alternative option is to provide a combination of B-raf proto-oncogene (BRAF) inhibitors and Mitogen-activated protein kinase (MAP2K1, otherwise known as MEK) inhibitors to those patients with somatic V600 mutations in the BRAF gene of the melanoma.[6–9] Between 40%

and 50% of patients will achieve a significant response to this latter treatment combination where V600 BRAF mutations are present.[8 10] MEK follows BRAF in the MAPK intracellular signalling pathway.[11] V600 BRAF mutations, that is, to say a mutation resulting in substitution of valine at position 600 in the amino acid sequence, results in constant signal transduction and promotion of cell growth and division.[11] The ability to rapidly test somatic tissue for BRAF mutations is of paramount importance in deciding who is eligible to access these treatments and for those with widely disseminated disease timeliness is a key determinant of quality care and cancer outcomes.[12] Healthcare systems that rely solely on next-generation sequencing (NGS) may not be able to meet the clinical needs of patients with stage 4 disease who could deteriorate within a short space of time. Patients can have profound clinical response within days and therefore knowing the BRAF status and being able to start BRAF directed therapy can make the difference between life and death. There is clear, real-world evidence from the setting of lung cancer that delays in somatic mutation testing can lead to missed opportunities for treatment intervention.[13]

In this study, we provide in-house verification data in support of service development of testing in local histopathology laboratories, discuss tissue requirements and provide a suggested tissue workflow for maximum efficiency of testing in the best interest of patients. We show that tissue samples with tumour nuclear content (TNC) below 50% are suitable for reliable use in the Idylla instrument contrary to the manufacturer's guidelines.

Aims and objectives

This project is aimed at developing our histopathology service to cater for melanoma patients' needs in an era of precision medicine. We aim to show that the concordance of BRAF testing in melanoma patients is of sufficiently high standard using automated PCR in comparison with NGS to provide a robust salvage pathway in patients with urgent clinical need for a rapid report.

We sought to verify minimum safe tissue requirements for rapid PCR-based testing using Idylla. The manufacturer guidelines currently recommend a minimum TNC of 50% for their rapid BRAF mutation cartridge.

The data will be used to demonstrate the automated PCR platform is fit for purpose in our laboratory. Limited workforce during the pandemic has reinforced the need to develop services that are automated with minimal staff input time. Work force constraints a have been shown to be an issue in National Health Service diagnostic pathology laboratories across the UK.[14] The Idylla platform was selected with this in mind. The cartridge format and automated DNA extraction means that it only takes 2–3 min of biomedical scientist (BMS) time to

perform the test.[13]

Methods

Patients with skin and mucosal malignant melanoma >1 mm thick or those with advanced stage metastatic disease were included in the study. Both primary melanoma and metastatic melanoma deposits in the form of formalin fixed paraffin embedded (FFPE) tissue samples were included for BRAF testing. BRAF mutational status in tumour metastases has been shown to be concordant with original primary tissue sample in more than 95% of

cases.[15] BRAF analysis was performed at request of the specialist skin multidisciplinary team meeting (MDT). One hundred patients whose requests pass through the histopathology department in Swansea on a prospective all comers basis were included. Requests were recorded by designated BMS staff and highlighted to our molecular team for Idylla BRAF testing. Standard of care requirements were met before verification testing.

All histological tissue specimens containing malignant melanoma >1 mm thick or specimens comprising metastatic malignant melanoma were tested for BRAF after tissue had been cut

for send away NGS panel testing at a centralised laboratory. All patients were recruited in sequence and verification data collected prospectively.

The manufacturer instructions state that >50% malign cellularity is required for the test to be valid but we tested all eligible patients to see if it were possible to reliably use the system in urgent cases. TNC in tissue sections was estimated according published guidance and based on ratio of tumour nuclei to background wild-type tissue nuclei.[16] Care was taken not to over- estimate TNC in specimens with dense chronic inflammation or in lymph nodes where wild- type nuclei in lymphocytes are small. See figure 1 which illustrates this concept using human tissue sections of metastatic malignant melanoma in a lymph node. Specimens with less than 50% TNC were marked for macrodissection to enrich the tissue sample for tumour cells was conducted on the molecular grade clean guide plate of the microtome. A dedicated microtome with clean space was used for 10 micrometre tissue curls to be cut for use in the dedicated cartridges sponsored by Biocartis. Use of a water bath in tissue processing was prohibited to avoid contamination of the cartridge with foreign DNA. A new microtome blade was used for each case tested.

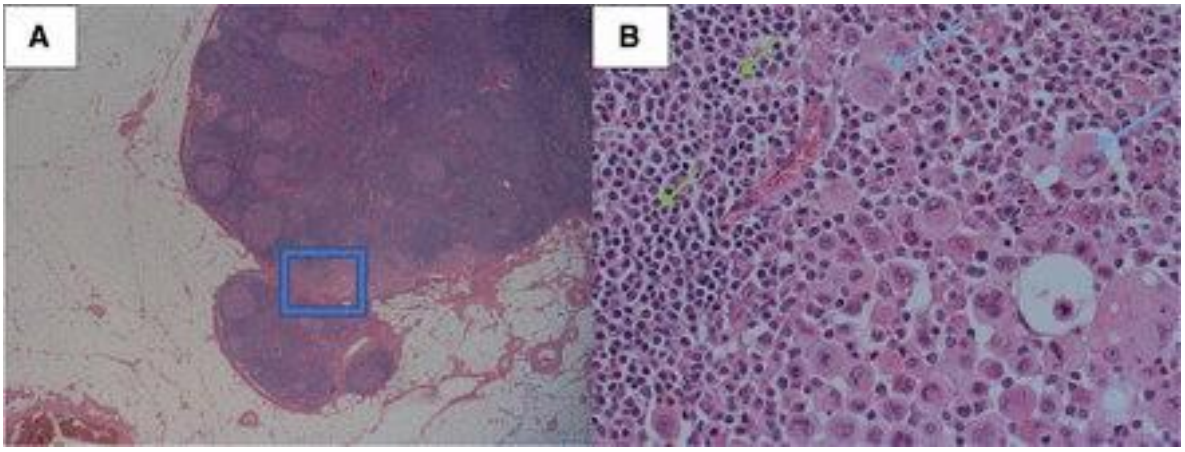


Figure 1

(A, B) Estimating tumour nuclear content.

Results were tabulated in an excel spread sheet against the specimen number only. No patient information was recorded to maintain anonymity. Patient consent was not required as this verification analysis examines an existing assay with known implications using tissue left over after the diagnostic process is complete as described above.

Testing platforms

Idylla BRAF mutation test

Idylla automated PCR by Biocartis using malignant melanoma solid tumour FFPE samples.

One hundred cartridges were used prospectively on an all-comer intention-to-test basis.

Requesting for BRAF mutational analysis was made according to standardised protocols via skin MDT team members to the laboratory. The Idylla BRAF mutation test is a real time PCR, qualitative in vitro test for the detection of V600K/R/M and V600E/E2/D mutations in codon 600 of the BRAF gene. At the DNA level the protein change corresponds to c.1798_1799delinsAA, c.1798_1799delinsAG, c.1798G>A and c.1799T>A, c.799_1800delinsAA, c.1799_1800delinsATor AC, respectively. The Idylla BRAF Mutation test requires more than 50% tumour neoplastic content (TNC) in a single FFPE section between 5 and 10 µm in thickness and is said to have an analytical sensitivity of 1% mutation detection in a background of wild-type DNA.[17] Macrodissection is recommended to increase

TNC following morphological assessment by a General Medical Council (GMC) registered cellular pathologist. The test has a turnaround time of 90 min and can be performed in any cellular pathology laboratory with 2 min of hands-on technical staff input time. All DNA extraction is automated within the Idylla BRAF mutation cartridge and testing platform. Quantity of DNA input was assessed by CQ value. Test outputs via console were as follows: no mutation detected; mutation detected in BRAF codon 600; insufficient DNA input; or invalid. Reported BRAF mutations were grouped as either V600E/V600E2/V600D tested in chamber B of the cartridge, or V600K/V600R/V600M which are detected in chamber C of the cartridge. The limit of detection for chamber B is 4 copies of mutant DNA and for chamber C 10 copies.[17]

Next-generation sequencing

NGS for somatic mutations in BRAF were conducted by an external laboratory for comparison with the Idylla BRAF mutation test. The external laboratory NGS test uses the Illumina TruSight Oncology 500 High Throughput DNA/RNA assay on an Illumina Novaseq6000. This DNA panel test screens hotspots in exons 11 and 15 to include coverage of codon 599, 600 and 601 variants in addition to hotspot regions of NRAS (exons 2, 3 and 4) and KIT genes (exons 9, 11, 13, 14 and 17). The key performance indicator for the laboratory turnaround time is 10 days from receipt of samples (60 μm of tissue on air dried, unstained glass slides) in their laboratory. This turnaround time does not include transit and slide preparation time therefore. The external laboratory provides a pyrosequencing service to reduce the turnaround time for reporting BRAF mutations to 7 days from receipt in their laboratory. The external laboratory test has a sensitivity of 98.8% and specificity of 99.2% for a minimum of 40 ng extracted tumour DNA.

Data collection and storage

Access to patient data is limited to Swansea Bay University Health Board staff. Technical support is available from Biocartis on request. Biocartis have no access to this data. The Idylla machine records data based on cartridge number. Additional support interpreting cases of sub-threshold mutations is available on a case-by-case basis. Mutation curves with low quality DNA can be studied but will not automatically be reported unless they have a CQ value of more than 40.

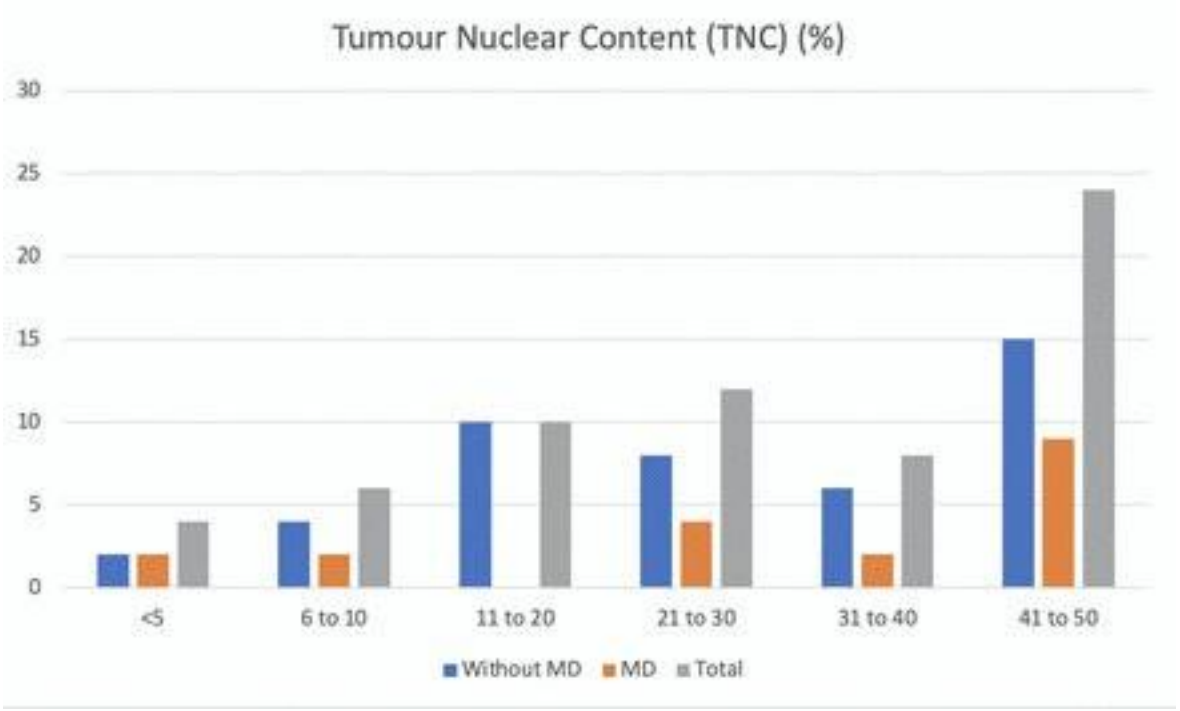
Germline mutations in BRAF are not being tested in this study which focuses on somatic solid tumour testing in an oncological setting of melanoma only to inform oncological treatment decisions.

Patient results from Idylla were not available for release to oncologists or patients before verification was completed and assessed satisfactory. This provided an ethical dilemma

for pathologists and biomedical staff where patients were known to be deteriorating and the potential for intervention became apparent through Idylla testing. The Idylla system is validated and certified as Conformité Européene In-vitro Diagnostic Device (CE-IVD) for clinical diagnostic use in the UK by the Medicines and Healthcare Products Regulatory Agency (MHRA). The lack of ability to act on BRAF results that may have impacted patient outcomes caused stress to laboratory staff.[18]

Results

A total of 100 patient tissue samples were tested using the Idylla BRAF mutation test following standardised protocols for NGS requirements. Fifty-one samples were macrodissected by referring to a marked tissue section stained with standard H&E (figure 2). Macrodissection increased TNC input for testing and the remaining samples could not



bemacrodissected (n=22) due to the distribution of tumour cells within the tumour sample, for example, multiple small foci of metastatic melanoma in a lymph node. The tumour neoplastic cell content ranged from <1% to 95%. All tumour percentages were assessed by one individual (AF) to ensure standardisation and remove potential for interobserver variation.

Figure 2

Bar chart demonstrating the number of tissue samples of TNC <50% that underwent macrodissection. MD, macrodissected.

A mean hands-on sample preparation time by technical staff of 4 min was recorded. Valid,"No mutation", test results (wild-type BRAF) generated CQ values were up to 34.4 for

our data set. Two samples with invalid, no mutation test results had CQ values of 36.2 and 36.4.

The Idylla BRAF mutation test failed to produce a test result on four occasions and required repeat testing. The wild-type content of BRAF was too high for detection of mutant alleles in two samples of the non-macrodissected tests. One was repeated and showed concordance with the NGS comparison. It was not possible to repeat the second as there was insufficient tissue remaining but there was sufficient DNA in the following macrodissected sample and this showed concordance with NGS results. A final tally of three failed results was returned for the cohort and these were all samples of primary skin excision for melanoma had TNC assessed as 10%, 30% and 40% (See tables 1 and 2).

Table 1

Performance of Idylla BRAF mutation test according to tumour nuclear content

	Estimated tumour neoplastic cell content			
	<1%–24%	25%–49%	50%–74%	75%–100%
Mutation detected	14	15	21	9
No mutation detected	17	6	28	34
Discordant NGS result	2	0	0	0
Insufficient DNA	0	1	0	1

- BRAF, B-raf proto-oncogene; NGS, next-generation sequencing.

Table 1

Performance of Idylla BRAF mutation test according to tumour nuclear content

Table 2

Breakdown of test characteristics for samples of 50% tumour nuclear content (TNC) or less

TNC	<5	6–10	11–20	21–30	31–40	41–50
Without MD	2	4	10	8	6	15
MD	2	2	0	4	2	9
Mean tissue area (mm^2)	330	368	395	259	344	329
Mean no 5 μm tissue sections used	1	1.16	1	1.54	1.11	1.13
Mean CQ	29.6	32.65	33.33	33.47	32.41	34.88
No result	0	1	0	1	1	0
% tests with mutated BRAF	25	40	37.5	83.3	37.5	50
Total no tests	4	6	10	12	8	24

- BRAF, B-raf proto-oncogene; MD, macrodissected.

Table 2

Breakdown of test characteristics for samples of 50% tumour nuclear content (TNC) or less

The majority of patients tested were male, n=61 and the remaining 39 were female. The mean

age of patients at the time of testing was 72 years (range 22–96 years). Two thirds of the tissue samples used for testing were primary sites samples (n=65) and the remainder were from metastatic sites (n=35). Primary sites included head and neck (n=15), limbs (n=26), back (n=16), chest/anterior abdominal wall (n=5). One melanoma occurred at a mucosal site. Recurrence at the primary site occurred in 29% of cases. The majority of metastatic sites were regional lymph nodes (n=15), followed by other skin sites (n=7) and a single case each of metastatic disease in the parotid gland, abdominal mesentery and oral mucosa (see table 3.

Table 3

Clinical characteristics of sample population

Characteristic		No=%
Sex	Male	61
	Female	39
Sample site tested	Primary	65
	Recurrence at primary site	10
	Metastatic site	25
Anatomical site of sampling	Head, face or neck	24
	Chest or abdomen	6
	Back	19
	Axilla, arm or hand	23
	Groin, leg, foot or toe	24
	Other	3
	Not stated on request form	1
Histological subtype of melanoma	Superficial spreading	38
	Nodular	28
	Lentigo maligna	2
	Acral lentiginous	0
	Mucosal	1

Table 3

Clinical characteristics of sample population

The histological types of primary melanoma reported in our case series were divided according to the current classification superficial spreading, nodular, lentigo maligna or acral lentiginous subtypes.[19 20] Most cases in our cohort were superficial spreading malignant melanoma (n=38), followed by nodular subtype (n=28), and lentigo maligna melanoma (n=2). No cases of acral lentiginous melanoma were represented. One case of anal mucosal malignant melanoma was identified. Ulceration was present on the surface of 37% of the melanocytic lesions.

Test performance

Overall concordance against standard of care testing was 95% as 5 mutations were detected by NGS outside of the probe set included in the Idylla BRAF mutation test cartridge.

However, the Idylla BRAF mutation test did show 100% concordance for the actionable variants V600E/E2/D or V600K/R/M within the cartridge primer set. V600E mutations are the most commonly encountered variant and occur in 75% of patients with a BRAF mutated melanoma.[21] The second most common variant is the V600K type which is found in a further 20% of mutated-melanoma patients.[22] The remaining 5% of patients tend to be point mutations in codon 600 resulting in amino acid changes to E2, D, R or M.[21]

The variants detected by NGS not available for detection by Idylla in this study were c.1397G>A p.Gly466Glu in exon 11 (n=1), c.1405G>A p.Gly469Arg in exon 11 (n=1), c.1801A>G p.Lys601Glu in exon15 (n=1) and c.1781A>C p.Asp594Ala in exon 15 (n=2).

The two melanoma samples with a Asp594Ala amino acid substitution are classified as variants of unknown significance in Clinvar[23] and, therefore, not being detected by the Idylla platform would have had no consequences for these patients. G466E, a rare mutation in exon 11 has been shown to be unresponsive to BRAF inhibitors in anecdotal case reports.[24] G469R in exon 11 is a class II kinase activating mutation in BRAF which has shown conflicting results in response to BRAF or BRAF +MEK inhibition with some patients not responding to treatment.[25] The BRAF exon 15 variant resulting in the amino acid substitution Lys601Glu (K601E) is a rare variant in melanoma that has been shown to respond to MEK inhibitor treatments such as trametinib in case reports.[26 27]

Discussion

The incidence of melanoma is reported to have increased by 140% since the 1990s in the UK and this increase appears to focus on males.[28] Incidence rates in males have nearly tripled whereas the rate has doubled in females.[28] This is perhaps reflected in our data set with 62% of our patients being male. Torso and limbs are the most common sites for melanoma occurrence and this is also reflected in our data.[28 29]

Two of the five rare BRAF variants detected by NGS that were not identified by the Idylla BRAF mutation test may have had clinical consequences for the patient if one relied on Idylla testing alone. The need to identify all possible mutations present in a somatic gene must be balanced with the need to detect the relevant mutations in a timely manner. Some authors advocate combined testing with Idylla and sequencing methods as a ‘belt-and-braces’ approach to detect the majority of melanoma BRAF mutations in 90 min but also allowing for detection of rare variants later on.[30] Many of these rare variants have limited data to support treatment but detecting them and publishing retrospective real-world data on responses to treatment will help to develop the evidence base for future practice and allow access to clinical trials. However, developing evidence for future use in melanoma care should not take priority over timely production of information regarding BRAF variants for which there is established evidence.

We know that more than 90% of BRAF mutations present as driver mutations in melanoma patients are the V600E variant which is readily detectable using the Idylla platform and showed a 100% concordance in our verification data. Experts agree that initiation of testing high-risk melanoma patients should be on a reflex basis by pathologists to prevent

delays in BRAF mutation identification.[31 32] However, the evidence is yet to emerge regarding the impact of reflex requesting BRAF analysis on survival outcomes.[33] There is evidence to show that starting BRAF targeted therapies while patients have a performance status of zero improves progression free survival and overall survival in advanced melanoma.[34] While there is no current data to predict which patients will deteriorate, it has been shown that there is an increased relative risk of 71% for death in those who commence treatment with a poor performance status.[34] Furthermore, patients with progressive advanced disease can show rapid clinical responses to BRAF inhibitors in less than 1 day.[35] We suggest an integrated clinical pathway to include rapid PCR testing using Idylla alongside NGS DNA panel testing, as outlined in figure 3.

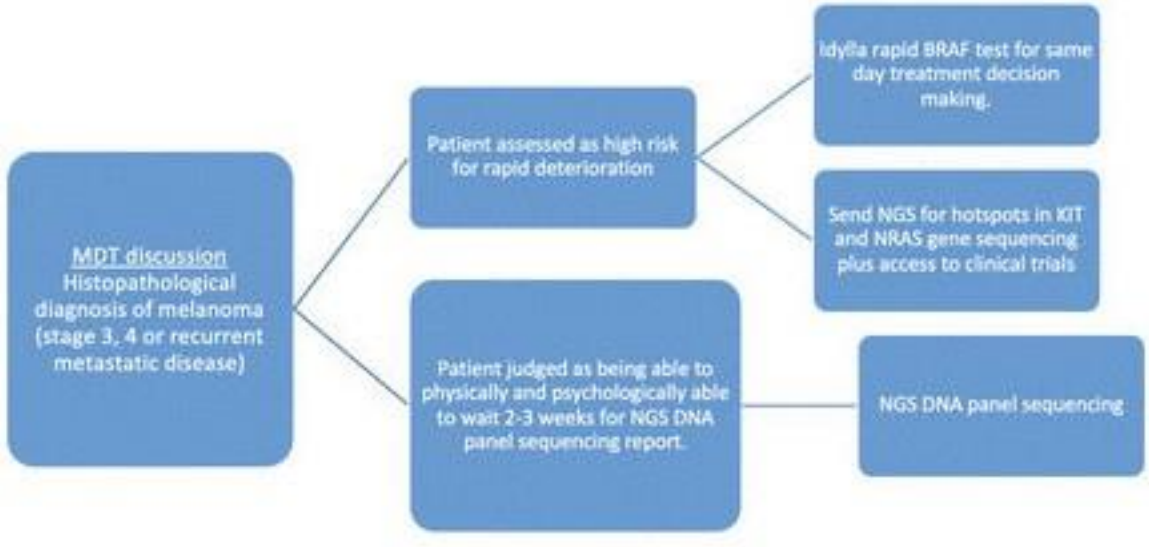


Figure 3

Proposed testing algorithm. BRAF, B-raf proto-oncogene; MDT, Multidisciplinary team meeting; NGS, next-generation sequencing.

The Idylla platform required much less tissue input in comparison to NGS. NGS requires

60 µm of tissue sections to be cut and sent to external laboratory for testing from our institution where NGS is not available in-house. Idylla BRAF mutation test requires a 5 or 10 µm paraffin curl only with no need to mount on glass slides. NGS, however, has a lower minimal input of tumour (≥10%) and Idylla BRAF mutation test requires more than ≥50% TNC according to the manufacturer's instructions. We have shown that the Idylla BRAF mutation test can effectively detect BRAF mutations in samples with TNC less than 50% (see table 2). Only 3 of the 64 (4.69%) samples with less than 50% TNC failed to produce a result. Being able to use samples with TNC <50% for testing increases the clinical utility of the Idylla

BRAF mutation test. The suggested testing pathway above (see figure 3) is designed to include all tissue samples. Just under 30% of the specimens with <50% TNC required macrodissection. While macrodissection increases the processing time marginally, scoring the paraffin block with reference to a marked H&E slide takes less than 10 min if the slides are marked by reflex action of the reporting pathologist. This compares favourably

with the time taken to prepare six 10 µm tissue glass slides and send to an external laboratory, no matter where in the UK they reside, not to mention the savings in consumables.

Our findings regarding TNC are in keeping with those of three studies which have documented successful use of tumour percentages below the validated range, in the region of 10% and above.[36–38] Like us, some have also had success with tumour neoplastic cell content (TNC) as low as 2%.[35] The CQ values achieved for the <5% TNC group in our study actually had the lowest CQ value of all those samples with TNC<50%. Lower CQ values reflect a reduced number of PCR cycles required for primer binding and detection of a mutation.

Furthermore, being able to use tumour sections with lower TNC% on the Idylla platform means a reduction in number of cases requiring macrodissection overall. The consequence of this is to potentially reduce the chance of false negatives from macrodissecting the wrong area of tissue, and also reducing the risk of DNA contamination. This study, which benefits from a large number of patients being tested, suggests that the recommended TNC% for BRAF variant detection using the Idylla BRAF Mutation Test should be altered in the manufacturer information sheet to reflect real world success in this patient group. While there is no reason to suggest that this is not also the case for other applicable tumour types such as thyroid neoplasms and colorectal cancer, we suggest that further real-world data be generated for specific tumour types other than melanoma for verification and to ensure compliance with medical laboratory standards of practice as dictated by ISO15189.

Conclusions

We conclude that the Idylla BRAF mutation test is a valuable tool in the histopathology laboratory molecular diagnostic tool kit with 95% concordance with NGS methods. There was 100% concordance for established variants for which there are well evidence-based treatments available. Testing in the histopathology laboratory will allow for reduced consumable use and time required for tracking and posting material to external laboratories for testing. In-house near patient testing in conjunction with NGS could benefit patients by allowing them to access treatments at an earlier time point in their disease treatment pathway and potentially increase likelihood of progression free survival.[34] Many clinicians argue that rapid somatic mutational testing is an ethical imperative to improve

outcomes for cancer patients.[39] We recommend that the Idylla BRAF mutation test be used on tissue sections with an estimated TNC of less than 50%. Furthermore, the real-world experience using the Idylla platform with a minimal hands-on time requirement for laboratory staff makes the system ideal for use in busy laboratories or where there may shortfalls in staffing due to the coronavirus pandemic.

Take home messages

- Clinically deteriorating patients with advanced melanoma can benefit from treatment with BRAF inhibitors on identification of a BRAF mutation in the histopathology biopsy/ resection specimen.
- Tumour section neoplastic cell contents of less than 50% generate valid BRAF results for clinical action using the Idylla BRAF Mutation Test. A turnaround time of 90 min for the Idylla BRAF Mutation Test has the potential to make a significant impact on patient progression free survival if it allows for treatments to commence before patients deteriorate to a performance status of less zero. A proposed testing algorithm is described. We demonstrate a concordance of 95% compared with next-generation sequencing (NGS).
- We hope this evidence will help support National Health Service Wales to adopt parallel testing of NGS for all possible variants in a panel of multiple genes alongside Idylla for rapid BRAF testing to facilitate prompt treatment of melanoma patients for enhanced survival outcomes.

Data availability statement

Data are available on reasonable request.

Ethics statements

Patient consent for publication

Not applicable.

Ethics approval

This service development project was peer reviewed by the Swansea Bay University Health Board Research and Development department (JSRC) and deemed as non-research. No identifiable patient information was collected, analysed or presented during this work. Tissue samples were tested using the rapid PCR test after conclusion of standard of care diagnostic procedures. Consent for use of limited amounts of archived FFPE tissues was not required according the the SBUHB R&D deportment review and in accordance with best practice guidelines from the Human Tissue Authority.

Acknowledgments

The authors would like to acknowledge the support of managers and technical assistance in slide preparation by laboratory staff in the cellular pathology department of Swansea Bay University Health Board.

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Footnotes

- **Handling editor** Runjan Chetty.
- **Contributors** AF conceived the project, wrote the study protocol for peer review by JSRC and secured testing apparatus. AF wrote the manuscript, performed part of the data analysis and is guarantor of the work. KM performed part of the data analysis, contributed to the manuscript and performed the Idylla testing. RDF provided clinical oncological context and contributed to the manuscript writing.
- **Funding** This is an investigator initiated study for service development purposes particular to Swansea Bay University Health Board. It was supported by Biocartis by provision of Idylla BRAF Mutation test cartridges. Biocartis did not seek to influence the purpose or design of this project nor did they have any involvement in the manuscript preparation.
- **Competing interests** None declared.
- **Provenance and peer review** Not commissioned; externally peer reviewed.

Appendix D

Title: The apparent "Inadequacy of PCR genotyping in advanced non-small cell lung cancer": A counter perspective.

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To the Editor:

Whilst not disagreeing with much of the factual content in your recently published letter by O'Sullivan et al, [1], we feel that their article fails to consider relevance in a wider clinical context whilst making unjustifiable comments/recommendations on the basis of a single (n=1) and atypical case report.

In summary, O'Sullivan et al, describe a lung cancer case with a novel deletion within EGFR exon 19 (L747_A755delinsSS), that was not detected in a tumour specimen using the Biocartis Idylla™ EGFR test but was identified using the NGS-based Guardant360® liquid biopsy test. The authors use this single case to highlight the "*Inadequacy of PCR genotyping*" and "*a significant limitation of the Idylla™ EGFR mutation test*", which after further mentioning the apparent "*futility*" of PCR based assays in relation to the detection of EGFR exon 20 insertions, they go on to "*recommend that in cases where there is adequate tissue for NGS, PCR-based tissue EGFR genotyping in isolation without parallel NGS be abandoned*".

Despite having ourselves published previously, both in relation to potential issues with PCR based tests and EGFR exon 20 insertions [4], and more generally regarding the considerable benefits of NGS-based analyses [5], we are concerned that readers not intimately

familiar with the technologies, may reach the unjustifiable conclusion that all PCR-based solutions, and especially the Idylla™, are 'inadequate' and that NGS potentially provides a near perfect alternative. Indeed, we feel that the article may be sufficient as to introduce undue alarm in NSCLC patients, or anyone managing such patients, whose treatment options have been guided using PCR-based assays.

By way of providing a counter viewpoint, we would like to highlight that whilst undeniably superior in many regards, how the benefits of NGS translate into what happens in the real world is more complex than it may initially seem.

To begin, the overall proportion of patients in which you may miss actionable EGFR mutations depends not only upon the test's sensitivity, but also the frequency of these mutations within the local population. This varies considerably worldwide, and whilst the authors concerns may be more valid in rarer subgroups with EGFR mutation rates ~50%, the effective impact in the UK for example, with incidence rates <20%, will be much reduced [5,6]. Secondly, although mentioning PCRs speediness, this also needs translating into real-world benefit; Finall et al, recently described how 6% of Stage 4 patients died before NGS results were reported, and further discuss the important contribution Idylla™ based testing can make within this cohort [7].

Citing our own (n=1) case report, we encountered a case (Q1 2022) where one of the commonest EGFR exon 19 deletions (Glu746_Ala750del at 82% VAF) was detected using NGS and reported 1 day after the patient died. This could have been detected and reported eight days earlier using the Idylla™, with the obvious potential for a different outcome. The sample was adequate and both technologies were available; however financial approval for parallel testing was not obtainable. We note that O'Sullivan et al. acknowledge that their patient was deteriorating clinically. Consequently, had they not had a novel variant and had Guardant360® results not arrived just in time, their own experience could easily have been reversed.

To best serve patients, we need suitable tools at our disposal, and we need to use them wisely with regard to cost and precious tissue. EGFR/KRAS (as G12C is actionable) testing, in very ill patients needs to utilise simple, rapid and cost-effective technologies, such as the Idylla™. Positive findings may be acted upon confidently and usually without further investigations. Although higher than with NGS, false negatives remain relatively rare and on a par with the performance of many tests considered entirely appropriate for clinical use. Although we agree with O'Sullivan et al, that if PCR proves uninformative, cases should be reflexed for NGS whenever possible. Service users should be aware of, but not unduly concerned by, the risks of false negatives if this isn't possible, and carefully evaluate the risks

of re-biopsy if being considered solely to enable further NGS analysis.

We disagree with the conclusions of O'Sullivan *et al*, with the apparent suggestion that PCR should always be discouraged in cases where it may mean there will be insufficient sample remaining for subsequent NGS. Firstly, clinical urgency must be considered as discussed, and secondly, such situations typically arise when both tests are undertaken in different laboratories. Returning to a tissue block to cut additional sections inevitably results in loss and thus should ideally only be done once in a specialised molecular pathology laboratory.

Whilst the Idylla™ is CE-IVD certified only for direct FFPE use, many have internally validated it for pre-extracted DNA input or use other PCR tests e.g. Cobas® EGFR (Roche). Alternatively, some will cut once, with sections going both into the Idylla™ and for DNA extraction. Certainly, within our own laboratory (PB), if potentially limiting we undertake a single extraction, then quantitate and apportion DNA between assays as required. Assuming that sufficient DNA was recovered for NGS initially (<5ng/µl in our laboratory), use of PCR- based analyses have not prohibited any reflex to NGS.

On a final technical note, although we suspect from experience [9], that L747_A755delinsSS was unlikely to have been detectable using the Idylla™, we note that the authors do not mention whether it was successfully identified by NGS within the same tissue sample, only in the subsequent liquid biopsy. This raises the possibility that a false negative outcome may have resulted either from use of a specimen with tumour cellularity below the minimum required and/or the patient may have had more than one primary tumour with the liquid-based biopsy detecting the variant in DNA shed from an alternative location.

In summary, integrated cellular-molecular pathology laboratories should be equipped, empowered, and appropriately funded to determine the best analytical approach depending upon the individual specimen/patient. For clinically unstable stage 4 NSCLC patients, we argue that available options must include upfront (not downstream 'salvage') access to rapid turnaround PCR-based solutions such as the Idylla™ EGFR test.

Funding: None

Conflict of interest statement:

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: P.B. reports honoraria from AstraZeneca, Bayer, Biocartis, Janssen, Novartis and ThermoFisher outside of the submitted work. A.F. reports travel expenses to conferences paid by Biocartis outside of the submitted work. G.G. reports honoraria from Eli Lilly and Merck outside of the submitted work. K.T. reports honoraria from

Roche outside of the submitted work. P.T. reports honoraria from AstraZeneca, Bayer, Janssen, Novartis, Pfizer, Roche, MSD, BMS, Agilent, Ventana and Guardant outside of the submitted work. Others declare no potential conflicts of interest.

Appendix E

RNA-based next generation sequencing in the somatic molecular testing of non-small cell lung cancer (NSCLC) in a centralized model: Real-world data to suggest it is time to re-consider testing options.

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Abstract: Best practice in the management of non-squamous, non-small cell lung cancer patients involves somatic testing for a range of molecular markers. Actionable oncogenic drivers of malignancy are increasingly being detected using RNA-based next generation sequencing in the UK by referral to centralized genomic laboratory hubs. Recent audit data from the authors case work has demonstrated an RNA sequencing failure rate of 35%. This article examines the real-world context which may account for this failure rate and discusses alternative options for patient care pathways.

Keywords: lung cancer; RNA sequencing; molecular pathology.

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1. Introduction

Lung cancer is the most common cause of death from cancer world- wide [1]. The United Kingdom (UK) has the some of the worst survival outcomes of developed nations across the world and Europe [1-3]. It is important for all healthcare professionals to understand factors that may be contributing to these poor outcomes for our patients. This commentary examines the role molecular biomarker detection may have in care of patients with non-small cell lung cancer (NSCLC) in the UK.

In the UK, patients are discussed at multi-disciplinary team meetings (MDT)(also known as tumour boards) to sense check diagnostic information from radiology and pathology with the clinical context and to determine the best management plan for each individual patient according to their wider health and circumstances [4, 5]. Predictive molecular biomarkers are needed to inform patient management options and consideration of targeted oncological agents [6, 7]. The array of biomarkers needed to inform the best management plan for non-squamous

NSCLC has evolved at pace in recent years [8]. At the time of writing, PD-L1 is performed by immunohistochemical methods providing sufficient malignant tissue is available, followed by somatic tumor mutations in Kirsten Rat Sarcoma Viral Proto-oncogene (KRAS), V-raf Murine Sarcoma Viral Oncogene Homolg B (BRAF), Epidermal Growth Factor Receptor (EGFR) by DNA next generation sequencing [9- 15]. Gene fusion events in Anaplastic Lymphoma Receptor Tyrosine Kinase (ALK-1), ROS Proto-oncogene Tyrosine-Protein Kinase (ROS-1), Neurotrophic Receptor Tyrosine Kinase 1, 2 and 3 (NTRK1/2/3), Ret Proto-oncogene, receptor tyrosine kinase (RET) and skipping lesions in exon 14 of the MET Proto-oncogene receptor tyrosine kinase (MET) can be identified using RNA-based next generation sequencing (NGS) in somatic tissue [16, 17)]. RNA sequencing is preferable to DNA sequencing for large structural rearrangements in somatic genomes where there are large intronic sequences in the DNA of the gene of interest, NTRK1 being a good example. [17, 18]. Sequencing spliced mRNA transcripts that consist solely of exons allows for more accurate detection of fusion events by current bioinformatic analytical methods [18, 19]. The incidence of gene fusion events in lung cancer is low [20]. ALK-1 rearrangements occur in approximately 3% of western populations with primary lung adenocarcinomas whereas ROS-1 is the cancer driver in less than 1% of cases [21]. RET and NTRK1,2 and 3 fusions and MET 14 skipping variants in NSCLC are also uncommon [15, 22-27].

Our local practice is to send tissue sections on charged glass slides to an external laboratory for molecular testing as our cellular pathology department lacks the molecular grade medical laboratory facilities and biomedical scientist (BMS) staff required to conduct DNA and RNA extraction from tissue. This process is best commenced at the same time the formalin fixed paraffin embedded (FFPE) tissue block is cut to prepare the haematoxylin and eosin (H&E) stained slide for morphological assessment by a histopathologist [28]. Cutting the FFPE block requires “re-facing” each time a BMS attempts to cut a section of tissue in order to ensure the surface is smooth and appropriately orientated to give a full slice representing all areas in the FFPE block.

This process of re-facing inevitably involves loss of small amounts of tissue for accuracy of slide production. Limiting slide processing to one single step and cutting all possible required slides up front to prevent waste is clearly an ideal step to prevent any valuable tumor tissue form being wasted [29]. This is no more important than in care of lung cancer patients where small samples such as bronchoscopic biopsies and endoscopic ultrasound guided (EBUS) fine needle aspiration cytology can yield very small amounts of tumor tissue (see figure 1) but with demand for a large amount of molecular information

required for diagnosis and treatment [30-32]. Pathologists with expertise in thoracic pathology are well advised to limit diagnostic immunohistochemistry use in such cases to just two protein markers, p40 and TTF1 for subtyping squamous and adenocarcinomas of primary pulmonary origin [4, 29].

This paper will examine factors to consider for best patient care in the predictive molecular biomarker identification in lung non-squamous NSCLC, adenocarcinoma being the most common type, and consider whether there is a need for change to current practice and how that might be achieved.

RNA sequencing: the real-world experience of a centralized model of clinical somatic testing.

Failure rate of RNA sequencing

Sending tissue sections to a centralized external laboratory is part of an agreed local care pathway. A "salvage" method was built into the RNA sequencing strand of the molecular biomarker pathway to address cases that fail to yield sufficient RNA. This salvage pathway requires additional tissue sections be sent upfront to the external laboratory upon RNA NGS request to mitigate against the extended time interval in requesting further tissue from the referring pathology laboratory.

An internal audit of the author's cases reported as adenocarcinoma of primary pulmonary origin between Nov 2021 and Jan 2022 (n=20), showed that RNA sequencing failed in 35% of requests. This failure rate in keeping with published experiences elsewhere in the UK [33].

Samples with insufficient material are not sent for RNA NGS and this is defined as samples with less than 100 malignant cells [34]. Such a high failure rate seems at odds with recently reported data regarding RNA- based next generation sequencing (NGS) from an Italian referral center that successfully produced results for 95.8% of their patient samples (n=48) using a customized gene fusion panel [16]. The assay validation study by de Luca, and colleagues, however, is not comparable with our experience as the authors only included cases where the desired RNA quality and quantity thresholds had already been met [16]. Another recent study of RNA-based NGS using cytology samples (n=129) processed into formalin fixed paraffin embedded (FFPE) cell blocks

found a success rate of 91% using a hybrid capture method of RNA sequencing [34]. Just 1 sample had insufficient RNA extracted and 8 were of insufficient cellularity [34]. The success of their method may relate to minimal fixation in 10% neutral buffered formalin (10 minutes). However, most tissue specimens submitted to cellular pathology laboratories are fixed in formalin for between 6 and 72 hours to meets standard operating procedures for quality in immunohistochemistry techniques [35].

Preanalytical considerations

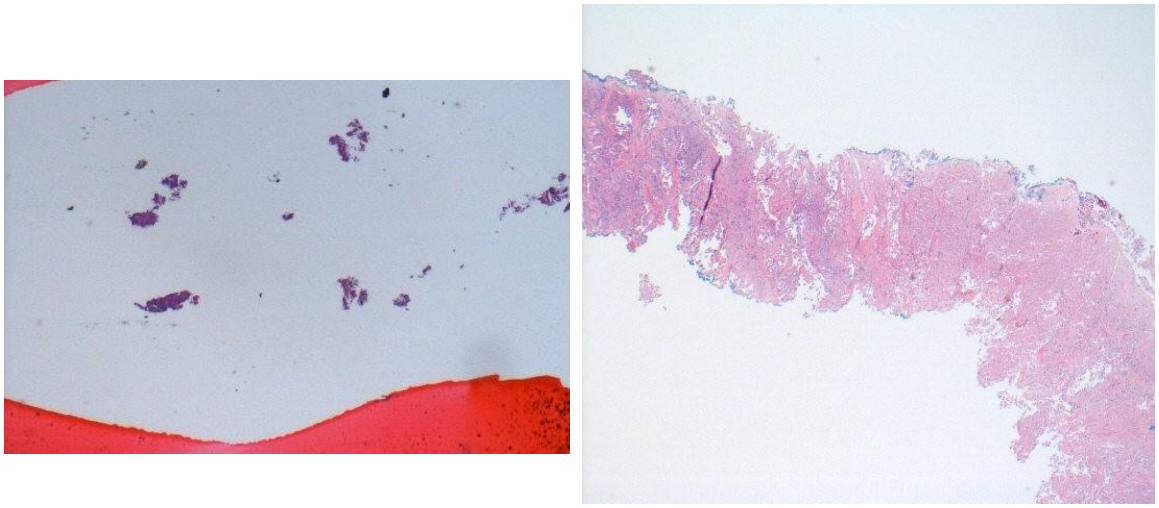
Formalin fixation causes cross linking of proteins within tissue to prevent tissue breakdown in archives [36]. Formalin causes direct degradation of RNA molecules and can also detrimentally interact with chemical agents used in RNA extraction [37]. RNA extraction is said to be more successful from fresh or frozen tissue samples rather than FFPE tissue samples, particularly if they have not been archived for long periods of time [38-40]. Clinicians may ask the question, “Why don’t we just move to using fresh tissue?’, which, on the face of it seems like a reasonable suggestion. That is, until one considers the huge logistical changes that would be required of histopathology laboratories to support such a change. It would involve change in practice by surgical theatre staff and porters. Some authors advocate using alternative

fixation methods to preserve tissue, such as the PAXgene solution [41- 43]. The morphological appearances of H&E tissue sections generated after fixation in PAXgene are excellent and comparable with FFPE [41, 42]. However, the costs of such a change would be prohibitive in a public funded, UK NHS setting. Five litres of 10% formalin costs in the region of £13 whereas 50ml of PAXgene (servicing small specimens only) will cost in the region of £150. The enhanced cost would also be compounded by a need to invest in new, dedicated tissue processors compatible for use with PAXgene in cellular pathology laboratories [44].

as a single-plex tool, offers the advantages of being fast, cheap, reliable and easy to perform on automated, large throughput platforms. Tissue consumption for each antibody is 3-4µm of FFPE tissue and offers the additional asset of marker assessment in a spatial context. That is to say, one can be sure that the protein biomarker of interest specifically relates to the malignant cells of interest and thereby enhances diagnostic confidence. That level of data granularity is lost in bulk sequencing assays using DNA or RNA extraction from tissue where there are nuclei with wild-type DNA and RNA species in connective tissue, inflammatory cells and normal background

epithelium within the tissue section [51]. See figure 1. Macro-dissection from the glass slide can help enrich samples for tumor nuclear content but this may not be possible in centralized molecular laboratories with no resident cellular pathologist expertise.

NGS offers the advantage of multiplex detection of molecular biomarkers but requires a much greater input of tissue for assessment than some rapid PCR assays available for clinical use. For example, the IdyllaTM Gene Fusion assay uses 5-15µm of FFPE tissue as compared with at least 50µm for each sequencing panel available to our patients [45]. When one considers that rescue FISH for failed RNA seq samples requires an additional 8µm of tissue per marker, not using such a rapid assay becomes difficult to defend in a setting of a small amount of available tissue [45]. The IdyllaTM Gene Fusion cartridge covers all the actionable gene fusion events for detection that may guide treatment decisions in lung adenocarcinoma patients [52-54].



(a) **(b)**

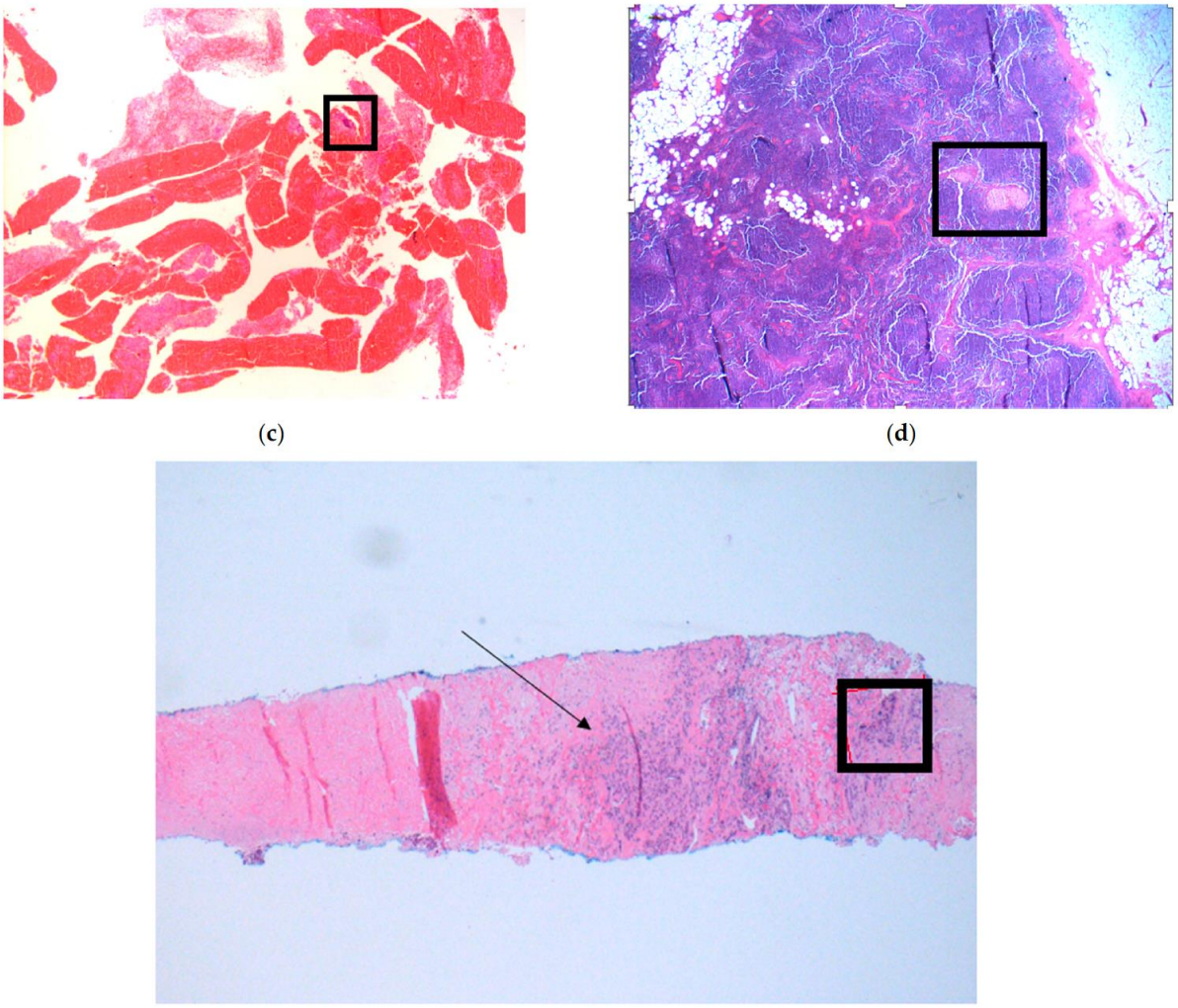


Figure 1. Photomicrographic examples of histopathological tissue biopsies containing scarce or no malignant tissue. (**a**) Photomicrograph of bronchial biopsy (x4 magnification) showing limited nature of some samples. This is small cell neuroendocrine carcinoma. Haematoxylin and eosin (H&E) stain; (**b**) Some CT guided core biopsies may not contain malignancy but rather necrotic material, as in this example. (x4, H&E stain); (**c**) Endobronchial ultrasound- guided (EBUS) fine needle aspiration cytology of mediastinal lymph nodes. EBUS samples often yield small amounts of tissue on a background of blood. A small fragment of carcinoma is highlight in the black circle (x4, H&E); (**d**) Photomicrograph illustrating a small deposit of metastatic carcinoma (black circle) in a lymph node. Bulk RNA extraction would from this section without, microdissection, is likely to yield large amounts of wild-type signal from lymphocyte nuclei; (**e**) An example of low volume malignancy with a CT-guided core biopsy of lung highlighted in the red square. There is background fibrosis and chronic inflammation present. (x4, H&E).

The option to test for NTRK fusions also applies to all other solid malignancies where standard oncological options have failed [55, 56]. It would be beneficial to utilize the infrastructure in place for NSCLC cases for the wider oncology patient community where appropriate. Beyond NSCLC however, it may be more cost effective to screen all solid malignancies by using NTRK immunohistochemistry before confirmation of positive findings using automated FISH rather than using the gene fusion cartridge by IdyllaTM as a first-choice method [57, 58]. The additional fusions of ALK-1 ROS-1, Met14 skipping and RET may not be indicated in malignancies other than NSCLC, so it may not be economically viable to use the IdyllaTM Gene fusion cartridge in this setting. NTRK immunohistochemistry is fast, cheap and consumes just 3- 4µm of FFPE tissue [58].

Further consequences of failed RNS NGS.

The consequences of not having a report of gene fusion events in NSCLC at the time of outpatient appointment with an oncologist is a waste of a valuable appointment slot and time of hospital staff. There are additional knock-on effects to consider such as patient dissatisfaction, anxiety, staff morale and, most importantly, missed opportunities to start effective treatments in patients at risk of rapid clinical deterioration [59]. As discussed, TKI treatments should be started in the therapy naïve setting [48]. In addition, if an opportunity to start TKI therapy is missed the patient loses an opportunity to receive an oral therapy in the community, an option that can relieve some workload of secondary care. The consequence is more patients wait for intravenous chemotherapy drug administration as day case patients in hospital facilities with limited capacity.

Why does RNA sequencing fail so frequently in our experience?

RNA sequencing can fail for many and varied reasons. Limited tissue and the impact of formalin fixation have been highlighted but little discussed is the specimen exposure to environmentally ubiquitous RNA degradation enzymes [60]. There are RNA degradation enzymes in the air, on our hands and work surfaces that can cause destruction of RNA within FFPE tissue sections. Indeed, it is surprising that RNA sequencing works at all given the

nature of the processing occurring in the histopathology laboratory upstream of the receipt by molecular lab. FFPE tissue specimens are cut in a large open room with no special sterile air flows or compartmentalization. Sections are floated on a water bath prior to mounting on glass slides with the inherent risk, though much guarded against, of contamination. Slides are then packed into a plastic, non-sterile

slide mailer and standard packaging for postage to the external laboratory by courier. These are less than optimal conditions for handling and preserving RNA for sequencing and may account for the reason why so many RNA NGS assays fail in our experience.

Practical alternatives to centralized NGS testing.

Genomic technologies are advancing in capability and at a pace beyond which NHS cellular and molecular pathology laboratories can evolve. As such, it is understandable that some molecular laboratories have invested in NGS for the advantage of being able to expand the repertoire of gene variants reported in a quick and responsive way without additional capital expenditure. Automated technologies are now available that can reduce the turnaround time for reporting NGS samples in a reduced gene panel in as little as three days. Sheffield, *et al*, showed they could generate biomarker NGS-based reports using the GenexusTM platform in as little as three working days [62]. Using this technique may represent an opportunity for local histopathology laboratories to incorporate fully automated NGS reporting alongside morphological and immunohistochemical data in one step [62]. This assay requires a minimum of 8 samples per run and this need for batching could have detrimental consequences for turnaround time in laboratories with small numbers of patient requests. This could be overcome by use of automated rapid PCR systems such as IdyllaTM which do not require batching. The IdyllaTM platform provides the ability to give clinicians same day biomarker results in urgent cases and could salvage outpatient appointments where NGS reports are not yet available [63]. The IdyllaTM Gene Fusion cartridge designed by Biocartis has the added advantage of being a multiplex assay [53]. A recent multi-center European study of the Gene Fusion assay obtained valid results in 98% of their patient in as little as 3 hours with good sensitivity and specificity [53]. This compares very favorably to our current experience of valid results in just 65% of patients using RNA-based NGS first-line.

Although fluorescence in-situ hybridization (FISH) is single-plex and potentially time consuming to conduct, this technique is well established and reliable for clinical use. There have been advances in FISH technologies in recent years with use of computer algorithms to count fluorescence signals with the effect of reducing turnaround times and human resource requirements of traditional FISH. This could be a very valuable adjunct to rapid PCR or immunohistochemistry (IHC) where used for screening out negative cases. Immunohistochemistry, being a rapid, cheap, fast and easily automated technique, makes it an ideal starting point for screening for uncommon gene fusion events given that it has a high negative predictive value in low incidence settings. This may be a particularly attractive approach for histopathology laboratories with well-established expertise in IHC practice and sufficient case throughput to justify testing by this method in the majority of cases. Certainly, in resource limited settings, such as the UK NHS, IHC should be considered as a robust option in NTRK testing for to all solid malignancies.

Suggestions for Improved Care Pathways Incorporating Molecular Biomarker Identification.

We have previously described an actionable oncogenic driver identification pathway for NSCLC patients that using rapid PCR for identification of common, known somatic mutations in stage 4 patients to prevent missed opportunities for starting TKIs in treatment naïve patients [45]. It may be best practice, however, to extend use of rapid PCR to assess for gene fusion events in all cases where molecular testing is indicated on small biopsies. This could prevent waste of valuable tissue given that, in our area, RNA-based NGS has a failure rate of 35% and the gene fusion cartridge has a much lower failure rate, in the region of 2%. [53]. See figure 2.

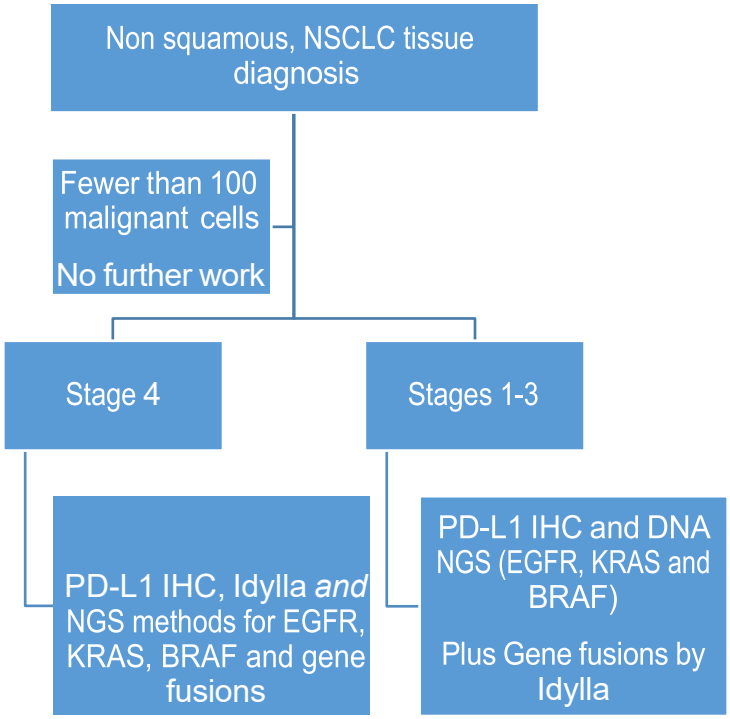


Figure 2: Suggested algorithm for molecular analysis of somatic NSCLC tissue taking into consideration the real-world failure rate of RNA sequencing performed in a centralized service model.

NSCLC, non-squamous, non-small cell lung cancer; PD-L1, programmed death ligand 1; IHC, immunohistochemistry; EGFR, epidermal growth factor; BRAF, B-raf oncogene; KRAS, K,ras oncogene.

Conclusions

Cellular and molecular pathologists working in the public sector have a duty to consider best use of often limited tissue samples to achieve maximum information for patient care. Pathologists, with the tissue morphology before them, are best suited to make the best choices regarding testing modality. Reflex requesting of biomarkers in NSCLC recognizes the role the pathologist can play in saving time for reporting of such biomarkers [64]. A recent audit of RNA sequencing reports a failure rate of 35%. There are a number of alternative

testing strategies to consider that could improve biomarker identification in NSCLC patients in our region, including FISH, rapid PCR and fully automated rapid NGS workflows that could be harnessed in-house with the added benefit of integration alongside morphological and immunohistochemistry findings in one report. Timeliness of reporting both cellular and molecular pathology findings in tissue biopsies is of paramount importance in the care of our lung cancer patients. Rapid near patient testing methods could positively impact up to a 1/5 of stage 4 patients and make a difference in overall progression-free survival [45]. This is of particular need in the UK where we lag behind our European colleagues who have a wider range of molecular testing capabilities at their fingertips and greater control over choice of testing

method based on individual patient needs [2, 3, 53]. However, whether the pathologist holds the key to closing the gap in survival outcome data for lung cancer patients in the UK remains to be seen. Pathologists should at least be given the opportunity to try.

Author Contributions: Conceived and written by Dr Alison Finall. Audit data reported in this article was collected and analyzed by Dr A Finall.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable. The study did not require ethical approval.

Informed Consent Statement: Not applicable.

Data Availability Statement: Available on request.

Conflicts of Interest: The author has received support from Biocartis to attend international conferences as a speaker. Biocartis had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish.

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Appendix F

Analysis of somatic epidermal growth factor receptor (EGFR) mutation by rapid polymerase chain reaction (PCR) using intra-operative frozen section tissue in early stage, non-small cell lung cancer patients.

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Abstract

Introduction

Osimertinib is now licensed for use in early-stage non-small cell lung cancer patients (NSCLC) as an adjunct in the post-surgical setting. Identification of appropriate patients for this therapy relies upon identification of somatic epidermal growth factor receptor (EGFR) mutations in exons 19 and 21. The Idylla™ EGFR Mutation Test is a rapid, fully automated near-patient test that could be used in histopathology laboratories to identify such patients.

Methods

We conducted a pilot study of 12 consecutive patients with non-squamous NSCLC histology undergoing intra-operative frozen section diagnosis over a two-year period to determine the suitability of frozen tissue for EGFR testing as compared with matched formalin-fixed paraffin-embedded (FFPE) tissue samples.

Results

There was 100% concordance of findings between the Idylla™ EGFR Mutation Tests conducted on frozen section and FFPE tissue samples. There was also full concordance with next generation sequencing (NGS) results where performed. The cycling quotient (CQ) value for fresh frozen tissue samples was significantly lower than that for FFPE samples ($p<0.0001$).

Conclusions

This is the first study to assess suitability of DNA from fresh frozen samples at time of intra-

operative frozen section for NSCLC patients using a rapid, automated, singe gene polymerase chain reaction (PCR) method. This test could be used to identify appropriate patients for post-surgical, adjuvant Osimertinib therapy. There are potential cost and time savings by choosing this singe gene test rather than utilising NGS methods for early-stage NSCLC patients.

Introduction

Standard of care for early-stage, non-squamous non-small cell lung cancer (NSCLC) patients (stages IB to IIIA) is surgical lung resection with lymph node dissection in those who are well enough (189). Up to half will develop disease recurrence after surgery with curative intent (190). The ADUARA trial showed that patients with somatic epidermal growth factor receptor (EGFR)-mutated early-stage NSCLC receiving adjuvant Osimertinib had longer cancer free survival times (191). Drug regulatory agencies across the world have recently licensed adjuvant Osimertinib for early stage, completely resected EGFR-mutated (exon 19 deletions and L858R mutations) NSCLC on the basis of this evidence (42).

More than one third of patients fail to receive a pre-operative tissue diagnosis prior to surgery due to inaccessibility or high-risk of serious morbidity as a consequence of an invasive biopsy (192). These patients have an intra-operative frozen section at the start of surgery to confirm malignancy and the need to proceed to surgical resection (192). The aim of this study was to determine whether a fully-automated, rapid polymerase chain reaction (PCR) assay could be used at the time of intraoperative frozen section diagnosis to support timely oncological management decisions.

Methods

Case selection

Consecutive adult (>18 years) patients undergoing intra-operative frozen section diagnosis of a lung lesion were included in the study. Rapid PCR was performed on the frozen section tissue after identification of morphology suggesting a diagnosis of adenocarcinoma, favouring lung primary or carcinoma, not otherwise specified (NOS) or by a cellular pathologist. Patients suspected of having tuberculosis are not eligible for frozen section and were excluded. Minimum sample requirements for rapid automated testing were 10% tumour nuclear content (TNC). Samples were frozen to -40^{o}C using the automated PrestoCHILL (Milestine Medical) platform with MMC, Milestone^{TM} Cryo-embedding Compound. An adjacent fresh tissue sample was selected for processing into a formalin fixed paraffin embedded (FFPE) tissue block for use as the paired patient sample.

Pre-analytical considerations

FFPE processing required fixation in 10% neutral buffered formalin for at least 6 hours prior to automated overnight processing on Thermo Fisher Scientific Exselsior ES tissue processors (2008). The oldest FFPE block used in the study dates from the end February 2020. Testing concluded by mid-September 2022. The maximum storage time for FFPE tissue blocks was 31 months.

Idylla ™ EGFR Mutation Test

Tissue sections were taken from the microtome direct to the 'Idylla™ EGFR Mutation Test' cartridge without use of a water bath or mounting on glass slides. Frozen section slides were assessed for TNC by a single pathologist (AF) according to published guidance (193). Tissue samples were sandwiched between filter paper discs moistened with nuclease free water to ensure adherence to test platform in the cartridge. Automated DNA extraction within the Idylla™ cartridge prevents sample contamination and need for molecular grade facilities within the histopathology department. Pre-determined primer sets identify the presence of up to 51 specific EGFR mutations within the assay by detecting fluorescence above a proprietary threshold during PCR. This primer set includes detection of 36 types of exon 19 deletion and the L858R mutation in exon 21 (3 variants). Cycling quotient (CQ) values were recorded as a proxy measure of DNA quantity. A CQ value of 20 is equivalent to 200ng of DNA as determined in experiments conducted by the manufacturer (96, 107). The rapid PCR assay has a CE-IVD certificate from the Medicines and Healthcare Products Regulatory Agency (MRHA) for use in a clinical diagnostic setting (107). The Limit of Detection of the test is less than 5% TNC (107).

Ethical considerations

The study proposal was reviewed the Swansea Bay University Health Board Joint Study Review Committee and deemed service development. Audit, service development and quality improvement projects are exempt from the need for research ethics committee review (194, 195). Compliance with Human Tissue Authority guidance on use of diagnostic human tissue is assured. Verification of all new assays require in-house verification to meet ISO15189 medical diagnostic laboratory standards.

Statistical analysis

Descriptive statistics and a paired t-test were prepared using SPSS V.26.0.0 statistical package from IBM and graphad.com. Study was closed when statistical significance for differences between CQ values between the paired sample types was achieved. CQ values

were assumed to follow a normal distribution.

Results

Twelve patients underwent frozen section with non-squamous, non-small cell neuroendocrine carcinoma (NSCLC) morphology. One of these frozen section samples was compromised and could not be tested as a fresh frozen sample. All samples submitted were peripheral lung wedge resections for primary frozen section diagnosis.

Most patients in the cohort had a performance status of 0 and a successful outcome following surgery. One patient was deceased 2 months after surgery due to cerebral infarction. See Table 1 for clinical details. None of our patients received adjuvant Osimertinib in the post-operative period. One patient started adjuvant Carboplatin treatment in a setting of regional lymph node metastases identified in the surgical resection specimen. Two cases were reported as carcinoma, (NOS) at frozen section; Both cases were poorly differentiated adenocarcinoma as determined by immunohistochemistry.

The Cycling Quotient (CQ) value is a proxy marker of the amount of DNA available for polymerase chain reaction (PCR). A low CQ value indicates that fewer cycles of PCR were required for sufficient levels of florescence to be detected for a valid call by the Idylla^{TM} instrument. Instructions for use state an optimal CQ value range of 19 and 24 for clinical reliability. The difference between the CQ values between the groups was determined using the paired T-test. See table 2. The two-tailed p-value is less than 0.0001, indicating a statistically significant difference. All eleven samples were devoid of EGFR mutation by rapid PCR and next generation sequencing (NGS). The outcome of the rapid PCR test was the same for FFPE samples and fresh sections samples in 100% of cases. The study commenced before Osimertinib was approved for clinical use in a post-surgical adjuvant setting in early-stage NSCLC.

Table 1: Clinical background, staging, treatment and follow-up status of patients undergoing intra-operative thoracic frozen section.

No.	Age years	Pre-op PS	Histology at FS	Histology at FFPE	Radiological pre-op stage and tumour size (mm)	Pathological Staging and tumour size	Osi Y/N	Follow up
1	73	0	Carcinoma poorly differentiated	Adenocarcinoma, solid pattern	T2a N0 35mm	T1c N0 28mm	N	Disease free and well @ 31 months post-op f/up
2	78	1	Adenocarcinoma Treated for TB	Adenocarcinoma, acinar	T2a N0 35mm	T1c N0 23mm	N	Disease free and well @30 months f/up
3	74	0	Adenocarcinoma	Adenocarcinoma, acinar. Foci x3 background AIS and x1 AAH.	T1b N0 15mm	T1a N0 10mm	N	Disease free and well @27 months f/up
4	75	0	Adenocarcinoma, mucinous	Primary enteric phenotype adenocarcinoma	T2a N0 17mm	T2a N0 (PL1) 34mm	N	Disease free and well @21 months f/up
5	70	0	Carcinoma NOS, poorly differentiated	Adenocarcinoma, poorly differentiated	T1b N0 15mm	T1a N1 10mm	N	Disease free and well @ 20 months f /up
6	79	0	NSCLC, favour adenocarcinoma	Adenocarcinoma, papillary 40% and lepidic 60%	T1c N0 21mm	T1b N0 20mm	N	Disease free and well @ 19 months f/up
7	80	2	Adenocarcinoma papillary and micropapillary	Adenocarcinoma, micropapillary 85%, and papillary	T2a N2 13mm	T1a N0 10mm	N	Deceased 2/12 post op from stroke
8	69	0	Adenocarcinoma	Adenocarcinoma, acinar	T1a N0 9mm	T1b N0 11mm	N	Disease free and well @ 9 months f/up
9	69	0	Adenocarcinoma	Adenocarcinoma, acinar and lepidic	T1b N0 12mm	T1b N0 11mm	N	Disease free and well @ 7 months f/up
10	74	1	Adenocarcinoma	Adenocarcinoma, papillary pattern	T3 (satellite nodules in same lobe) N0 27mm	T2a (PL1) N2 30mm Background granulomas related to aspiration.	N	Disease free and well @ 7 months f/up.
11	64	0	Adenocarcinoma	Adenocarcinoma, foetal type	T1c N1 26mm	T2a N0 31mm	N	Disease free and well @ 2 months f/up

Table 1: Clinical background, staging, treatment and follow-up status of patients undergoing intra-operative thoracic frozen section.

Table 1 Abbreviations: T, tumour; N, lymph node; PL, pleural invasion; EGFR, epidermal growth factor receptor; TPS, performance status; FS, frozen section; FFPE, formalin fixed, paraffin embedded; Osi, Osimertinib; No, case number; f/up, follow-up; TB*, Mycobacterium tuberculosis.*

Table 2: EGFR analysis using a rapid PCR assay and fresh frozen or formalin fixed paraffin embedded (FFPE) tissue.

No.	TAT for FS (minutes)	Sample size FS (mm^2)	C Q value FS	Sample size FFPE (mm^2)	C Q value FFPE	TNC (%)	Mutational status of EGFR FS	Mutational status of EGFR FFPE	Details of Next Generation sequencing
1	28	10 µm 180mm	16.6	5µm 228mm	22.6	10	No mutation	No mutation	ND
2	37	5µm 414mm	16.2	5µm 420	24.8	40	No mutation	No mutation	ND
3	51	10µm 126mm	16	5µm 300	25.5	5	No mutation	No mutation	ND
4	33	NR	17.1	5µm 255mm	24.6	10	No mutation	No mutation	1.Insufficient DNA (FFPE) 2. Repeat, **EGFR WT** BRAF, KRAS,NRAS, PIK-3CA, CDNKN2A, ERBB2, PTEN, RET also WT.
5	47	10µm 48mm	16.1	10µm 100mm	25.1	15	No mutation	No mutation	**EGFR WT** as full panel. above RNA seq negative.
6	52	5µm 320mm	15.8	5µm 580mm	22	25	No mutation	No mutation	ND
7	20	5µm 130mm	15.7	10µm 25mm	24.1	30	No mutation	No mutation	ND
8	73	5m 270	14.5	10µm 25mm	21.7	40	No mutation	No mutation	ND
9	49	5µm 320mm	15.4	5µm 170mm	22.3	30	No mutation	No mutation	ND
10	42	5µm 300mm	14.7	5µm 522mm	23.2	20	No mutation	No mutation	**EGFR WT** BRAF WT KRAS 34G>T exon 2
11	40	5µm 300mm	14	5µm 528mm	19.9	40	No mutation	No mutation	**EGFR WT** BRAF WT KRAS WT

Table 2: EGFR analysis using a rapid PCR assay and fresh frozen or formalin fixed paraffin embedded (FFPE) tissue.

Abbreviations: EGFR, epidermal growth factor receptor; TAT, turn-around time; FS, frozen section; FFPE, formalin fixed, paraffin embedded; TNC, tumour nuclear content, CQ, cycling quotient; ND, not done; WT, wild-type; NR, not recorded.

Discussion

Our study is the first to assess frozen tissue for molecular analysis of NSCLC tissue during intra-operative frozen section diagnosis specifically using the Idylla™ EGFR Mutation Test. Intra-operative molecular analysis has been trialled in breast cancer patient to refine diagnosis of metastatic disease within axillary sentinel lymph nodes with good results but a role for molecular diagnostics has yet to be demonstrated in other clinical settings (196, 197).

Our data suggest that the quantity of DNA available for PCR in the PCR assay was greater in the fresh frozen tissue samples than in matched FFPE tissue samples. This is an expected finding as formalin-induced protein-DNA cross linking requires harsh chemical

treatment to liberate DNA for PCR. The process of DNA extraction from FFPE tissue results in single strand breaks and smaller nucleotide length, as would be reflected in the CQ value (198).

Despite this the rapid, fully-automated PCR assay was designed for use in lung cancer specimens in standard histopathology practice and based on FFPE tissue validation protocols. As such, the instructions for use indicate a recommended CQ spectrum of between 18 and 25 for wild-type EGFR DNA (107). The mean CQ value found for FFPE was 23.225 and for fresh frozen tissue 15.645 in our paired samples set and this was a statistically significant difference ($p>0.0001$). FFPE results were well within the expected CQ range. Most studies agree that the quantity and quality of extractable DNA is superior in fresh frozen tissue compared with FFPE archived tissue (196, 197, 199-203) and this is clearly reflected in our data. The only other study to assess frozen section tissue use in an Idylla^{TM} assay focussed on the GeneFusion assay in work by Sorber, *et al* (179) . However, our findings contrast with those of Sorber, *et al,* who describe deterioration of nucleotide content when comparing paired frozen/FFPE lung malignancy samples. Their study focussed on RNA content and used frozen tissue samples that had been stored for up to 9 years at -80^{o}C prior to analysis (179). RNA is a more fragile nucleotide compared to DNA. RNA can suffer degradation from nuclease enzymes in the environment and is sensitive to hydrolysis because of the additional hydroxyl group in its ribose structure (204). The number of frozen section diagnoses taking place in our institution was substantially reduced by restrictions to operative practice during the COVID-19 pandemic and is a limitation of our study. A further limitation of our work is not having access to molecular laboratory facilities to extract DNA from the paired tissue samples to directly quantify DNA content (205). The decision to treat with Osimertinib relies on information from the EGFR gene alone and so this represents a clear opportunity for single gene testing by PCR. This approach could save considerable amounts of money by refraining from a blanket approach of DNA NGS sequencing for all lung cancer patients. The single gene test is around £150 per sample as opposed to £785 (Illumina Trusight^{TM} NGS panel) per sample cost (43). Cost and time efficiency are important considerations for all patients treated in resource-constrained health care services.

Conflicts of Interest

AF declares receipt of financial support to attend international medical conferences from Biocartis. Remaining authors declare no conflict of interest.

Author Contributions

AF conceived the idea and protocol and sourced testing cartridges. Testing was performed by KH and KM. AF analysed and interpreted the data and wrote the manuscript. IG identified patients for frozen section pathological diagnosis. GL, TE, IG and SB made significant intellectual contribution to manuscript drafts and all authors approved the final version for publication.

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