Molecular Diagnostics in Lung Cancer

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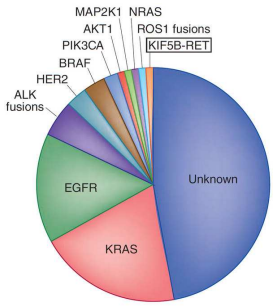
Oncogenic drivers in pulmonary adenocarcinoma



What is the surgical pathologists role?

1. **Diagnosis**

2. Classification
   - Small cell
   - Adenocarcinoma
   - Squamous carcinoma
   - Non-small cell carcinoma, not otherwise specified
   - Others

- **At present >90% of lung cancer patients have no actionable mutations so treatment is dependent on accurate classification.**

3. Assessment of suitability for molecular testing in appropriate cases
   - Need to understand requirements of local testing laboratory
   - Sufficient tumour / assess % tumour
   - Mark up sections for macro-dissection

4. Collation of results of molecular testing and histology / cytology report
   - Linked reports or supplementary reports appended to original histology / cytology report

5. Particiaption in EQA schemes.
   - NEQAS
   - European schemes

Organisation and Laboratory Issues

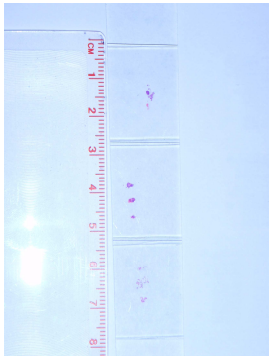
- Tissue fixing and processing
- Optimising use of tissue
- Prevention of cross contamination (tissue and DNA)

Optimal tissue processing

- Fixation schedules
  - Ideally 6-48 hrs depending on tissue size and type
    - Longer or shorter times may be associated with poorer quality DNA
  - Where should it occur?
  - Do we need to look at invest in vacuum systems to transport tissue and then control fixation in the lab?
- What fixative?

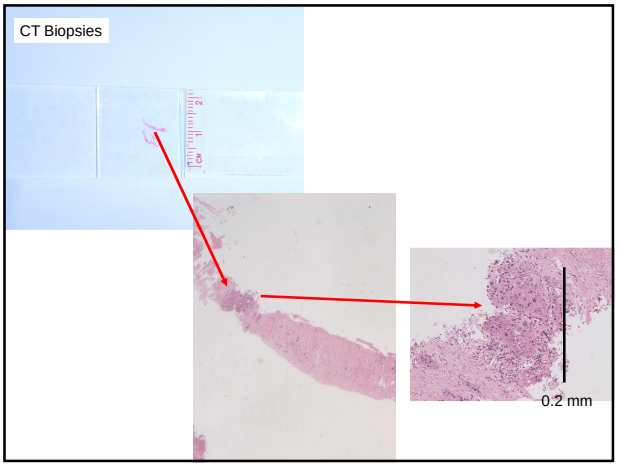
Ensuring optimal handling, fixation and processing of all specimens submitted for diagnosis (both prior to receipt of the specimen and within the lab).
- Cree IA etal. Guidance for laboratories performing molecular pathology for cancer patients. JCP Online First July 10, 2014 as 10.1136/jclinpath-2014-202404.

Optimising use of tissue



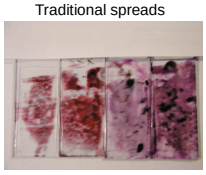
- Bronchial Biopsies
  - In general around 2mm fragments
  - Less than half of fragments contain any tumour at all
  - Median area of tumour within tissue 28%

Coghlin CL et al J Thorac Oncol 2010;5:448

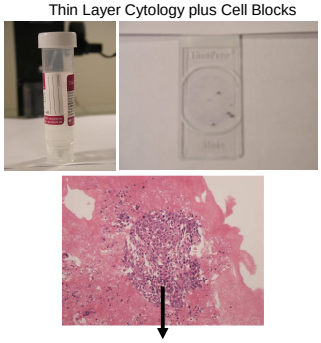


Cytology

Traditional spreads



Thin Layer Cytology plus Cell Blocks



Immunohistochemistry
PCR
FISH

As pathologists we need to:

- Educate radiologists and physicians on the fact that there is a difference between enough tissue for diagnosis and the amount required for full assessment!
- Ask BMS staff to clip up tissue cores/fragments in more than one cassette
- Request IHC only when required and only the minimum number of stains

In addition you may want to consider other strategies such as:

- Routinely cutting spares for IHC to reduce the tissue lost in 'facing up' blocks
- Use of double label IHC eg CK7+TTF1 and CK7+p40

Prevention of cross contamination

- Cross contamination of equipment
  - Use of fresh blades for cutting sections from blocks for pcr
  - Dedicated pcr cutting areas
- Accidental transfer of tiny tissue fragments (and ?DNA) between cassettes in processors and at embedding
- Identification of established procedures that work well but introduce contaminants into specimens

Cross-contamination in the Molecular Detection of *Bartonella* from Paraffin-embedded Tissues

M. Varanat, R. G. Maggi, K. E. Linder, S. Horton, and E. B. Breitschwerdt

Vet Pathol 46:940–944 (2009)

Table 1. PCR amplification results from swabs of 5 microtomes.

Sample Source	PCR Results*				
	M1	M2	M3	M4	M5
Negative Control (swab)	Negative	Negative	Negative	Negative	Negative
Platform	Negative	Negative	Negative	Negative	Negative
Block Holder	Positive	Positive	Negative	Negative	Negative
Slanting Platform	Positive	Negative	Negative	Negative	Negative
Used Blades	Positive	Negative	Negative	Negative	ND
Bench Top	Negative	Negative	Positive	Negative	Negative
Blade Holder	Negative	Negative	Negative	Negative	Negative
Extraction Control	Negative	Negative	Negative	Negative	Negative

*M = microtome; ND = not determined.

Occult Specimen Contamination in Routine Clinical Next-Generation Sequencing Testing

Jennifer K. Sehn, MD,[1] David H. Spencer, MD, PhD,[1] John D. Pfeifer, MD, PhD,[1] Andrew J. Bredemeyer, PhD,[1] Catherine E. Cottrell, PhD,[1] Haley J. Abel, PhD,[2] and Eric J. Duncavage, MD[1]

Am J Clin Pathol October 2015;144:667-674

After routine specimen processing 3% of extracted DNA samples contaminated by a minimum of 5% DNA from another individual
- occult floaters in the block?

Iatrogenic DNA Contamination!

Cell blocks with plasma-thrombin technique

- used world wide
- pooled plasma obtained from SNBTS
- sufficient free DNA to make sections from an 'empty' cell block pass a pcr DNA check
- foetal calf DNA gives a result which looks like a positive EGFR exon 19 deletion!

Current Tests in Routine Practice

- EGFR mutation testing
  - TKI inhibitors
- ALK translocation
  - Crizotinib / Ceritinib
  - KRAS mutations
  - BRAF mutations
  - ROS1 translocations

Trials – academic and commercial

Cancer Research UK
Matrix Trial

NSCLC Histology	Molecular Cohort
Squamous Cell Carcinoma (SCC)	FGFR mutation
Adenocarcinoma (ADC)	FGFR mutation
SCC & ADC	Liver Kinase (LK)-B1 mutation
SCC & ADC	Tuberous Sclerosing complex (TSC)-1/2 mutation
SCC	Retinoblastoma (Rb)-wildtype without homozygous loss of Rb) and homozygous p16 loss
ADC	CDK4 amplification
SCC & ADC	CCND1 amplification
ADC	KRAS mutation
SCC & ADC	MET amplification
ADC	ROS1 gene fusions
SCC	Neurofibromin (NF)-1 mutation
ADC	NF1 mutation
SCC & ADC	NRAS mutation

When to test

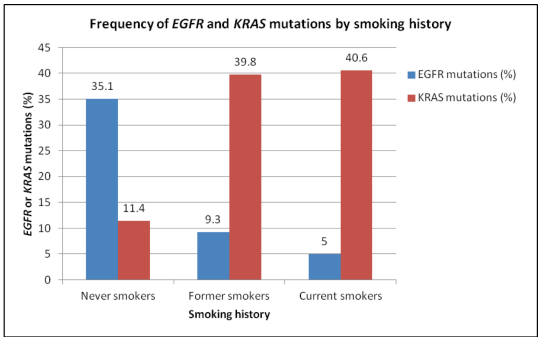
- **Have you enough tumour?**
  - All specimens should be assessed histologically / cytologically prior to testing
  - Macrodissection to optimise tumour content is essential
  - Depends on methods used – in our lab minimum specimen requirement is 10% tumour cells but ideally 20%

What to test (outwith clinical trials).

- Adenocarcinoma
- NSCLC (NOS)
- Adenosquamous carcinoma
- Any squamous carcinoma where there is a suspicion there could be glandular component
- ? Squamous carcinoma in young patients / non smokers

CAP/IASLC/AMP Guidelines
Lindeman NI etal J Thorac Oncol 2013;8:823-859.

- Patients should not be stratified for testing on the basis of
  - Gender
  - Smoking history
  - Ethnicity



- **Should we reflex test all cases (including lung resections)?**
- **Some will argue -Yes**
- **In my opinion – No!**
  - Why waste resources testing patients going to surgery or being treated with curative intent with chemo/rads?
    - Testing on diagnostic sample can be done at a later date or on a contemporaneous sample when required
  - Why test resected adenocarcinoma?
    - There is no evidence to use these drugs in the adjuvant setting
    - Testing can be done retrospectively or on contemporaneous sample obtained to confirm relapse.

- **'Intelligent testing'!**

  These drugs are used in the palliative setting.

  So:
  - Reflex test all patients who have advance stage disease on the basis of the specimen you have eg pleural aspirates, neck node FNAs, liver biopsies and biopsies of other distant sites.
    - These are the patients who are routinely candidates for these drugs
  - At MDT decide to test those where it becomes clear that the oncologists are considering the use of TKI or ALK inhibitors if mutation positive ie patients where palliative treatment options are chosen on the basis of stage, PS or some other consideration.
  - Have in place good liason with molecular pathology service to ensure good turnaround of cases. With good organisation testing results can be delivered in 3-5 working days. If requested and actioned at MDT results can be available before patient is seen in the oncology clinic

Suitablity of cytology samples for testing

No longer really controversial

Pleural fluid samples, EBUS / EUS FNAs etc all potentially suitable for testing and can give good results

Bronchial brushings and washing less commonly provide suitable material due to low tumour cell numbers and very low percentage.

Cytology specimens should be processed to a cell block for microscopic assessment prior to testing

- % tumour scores may be lower and macroscopic dissection not really an option

What is an adequate EBUS sample?

- Tumour cells often make a small percentage of total cells in an FNA.
- Any sample with cells will give a 'result'!!
  - Searching for small numbers of mutated genes in a large pool of wild type genes from large numbers of non-tumour cells.
- Wide variations in reported practice
  - > 40% Schuurbiers OCJ et al J Thorac Oncol 2010;5:1666
  - 20-40% Billah S et al Cancer Cytopathology 2011;119:111
  - >5% Betz BL et al Am J Surg Pathol 2011;136:564
  - ?? Navani N et al *Am. J. Respir. Crit. Care Med 2012;185:1316*
    - 'EGFR mutational analysis was possible in 107 (90%) of the 119 patients in whom mutation analysis was requested.'

    The 10% failures were technical eg insufficient DNA, amplification failure etc

    - but no indication of what the minimum tumour cell requirement for referral
    - no indication of how many cases were regarded as insufficient for testing and so this was not requested.
    - This paper has become interpreted in respiratory circles to imply that all malignant EBUS samples are therefore suitable for testing!!

Edinburgh experience of EBUS aspirates

Our laboratory 'standard' is 10% tumour cells as minimum.

42 aspirates from 32 patients with an EBUS cytological diagnosis of adenocarcinoma.

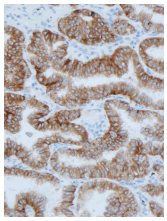
- ie these cell blocks contained sufficient material allow diagnosis and classification by IHC.

31/42 (74%) aspirates were regarded as suitable for testing

Equates to 24/32 (77%) of the patients had samples which were adequate for testing

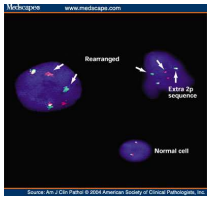
ALK testing – what to do?

IHC



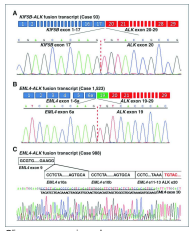
1. D5F3 Ab +/- Ventana kit
2. 5A4 Ab

FISH



Vysis break apart probes

rt-PCR



Clincancerres.aacrjournals.org

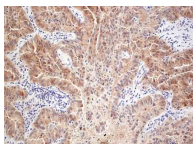
- IHC pos / FISH neg
  - May be related to polysomy
  - Reports that some my be positive by RT-PCR
  - Clinically some of these patients have been shown to respond to ALK inhibitors but experience limited
- IHC neg / FISH pos
  - The really contentious bit!

Cabillac F et al J. Thorac Oncol 2014;9:295-306
- Parallel testing IHC and FISH on 3244 NSCLC cases
- IHC or FISH positive in 150 cases (4.6%)
- Only 80 of the 150 positive by both
  - IHC neg / FISH pos 36
  - IHC pos / FISH neg 19
  - IHC pos / FISH fail 15

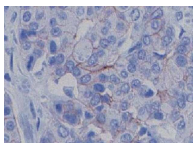
Rogers TM et al J Thorac Oncol 2015;10:661-618.
- 306 cases of NSCLC
  - IHC 100% sensitivity

Sullivan HC et al Appl Immunohistochem Mol Morphol 2015;23:239-244.
- 110 cases
  - IHC 100% sensitivity

- Currently three principal approaches to the IHC / FISH issue each with its supporters!!
  - FISH only
  - IHC + FISH all cases
  - IHC as a screen
    - If negative stop
    - If equivical or positive – FISH for confirmation



ALK IHC +

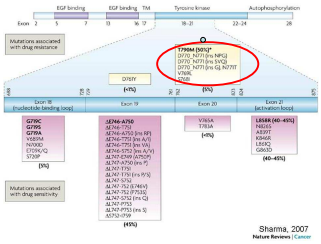


ALK IHC equivocal

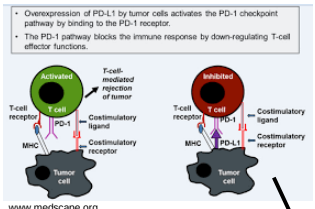
- rt-PCR
  - Role evolving
  - Offers potential way forward in cases where FISH equivical or fails
  - May allow ALK (and other translocations) to be tested for as part of multiplex testing.
- IHC only testing
  - May be an option with the Roche Ventana D5F3 Ab
  - kit which gives more binary (positive v negative) result but more difficult with other protocols as the proportion of FISH positives is low with weak staining.

Repeat EGFR testing

- EGFR resistance mutations may become apparent at relapse on TKI therapy
  - most common is the T790M in exon 20
  - MET amplification and 'drop out' of exon 14
- 3rd line TKIs available which may still have efficacy
- Need for repeat biopsy and testing at time of relapse
  - Potentially an increasing need as more options become available and 'current mutation status' is required
  - In many patients this may however not be an option given age and performance status
  - Role of cell free serum DNA screening

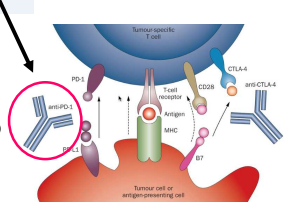


PD-L1 expression



www.medscape.org

Nivolumab
Pembrolizumab



Thasso.com

Assessment of the PD-L1 status by immunohistochemistry: challenges and perspectives for therapeutic strategies in lung cancer patients

Marius Ilie[1,2,3,4] · Véronique Hofman[1,2,3,4] · Manfred Dietel[5,6] · Jean-Charles Soria[7,8] · Paul Hofman[1,2,3,4]

Table 2 Key challenges regarding PD-L1 IHC for NSCLC patients

Biological	Dynamic induction of PD-L1 expression
	Intra-tumoural heterogeneity of PD-L1 expression
	Inter-tumoural heterogeneity of PD-L1 expression
	Unknown temporal evolution of PD-L1 (from resected to metastatic disease)
	The level of expression of PD-L1 can be modulated by anti-cancer therapies (radiotherapy, chemotherapy, targeted therapies)
	The level of expression of PD-L1 can vary according to the genomic alterations present in the tumour
	The level of expression of PD-L1 can vary according to the level of tumour hypoxia
Technological	The time for fixation in formaldehyde can modify the level of expression of PD-L1
	The different clones of anti-PD-L1 antibodies do not have the same affinity for PD-L1
	The different clones of anti-PD-L1 antibodies do not recognise the same epitopes on PD-L1
	The systems for amplification and detection of the signal change the threshold of positivity of the PD-L1 signal

- Expression of PDL-1 by IHC using a variety of different primary antibodies currently available in kits linked to specific platforms eg DAKO and Roche Ventana
- Different kits and scoring thresholds associated with Phramaceutical companies and their different drugs
- FDA and EMA have approved nivolumab without the need for IHC assessment of expression
- Pembrolizimab currently available for squamous and non-squamous carcinomas under early access scheme
  - Potential for huge demand for testing but currently very limited capacity
  - Should labs stick with expensive kits or are in-house tests using antibodies acceptable? If so how do we validate these?
  - What is the relationship between test results obtained with different kits?
  - What needs to be scored?
    - Tumour expression
    - Tumour infiltrating lymphocytes
    - Stroma

ISSUES IDENTIFIED

1. Occasional false positive EGFR exon 19 deletions

- 2 bp insertion / deletion mutations can be seen with Therascreen and Cobas ((c.2239_2240delinsCC [p.Leu747Pro]) or L47P
- Rare mutation but may be associated with resistance to TKIs
- We now confirm all these by Sanger sequencing

K Walsh, WA Wallace, R Butler, MJ MacKean, DJ Harrison, D Stirling and A Oniscu.A cautionary lesson on the use of targeted methods for *EGFR* mutation analysis: A case study. J Clin Path 2014;**67**:734-735.

2. Tissue volume and quality!!

- Respiratory medicine increasingly fixated on cytology.
- Just because we can do things sometimes on samples does not mean that we can achieve all that is necessary all the time!
- CRC UK SMP2 and Matrix Trial may help in demonstrating this issue and encourage oncologists to push for better samples.

What we are expected to deliver!

- 2006
  - ? Malignant
  - ? SCLC or NSCLC

→

- 2016
  - ? Malignant
  - ? SCLC or NSCLC
  - If NSCLC what is the cell type?
  - If not squamous can we have EGFR / KRAS etc mutational status?
  - Can we have ALK-EML4 / ROS1 gene rearrangement?
  - PD L1 expression?
  - Is there enough for this patient to be put in a clinical trial?

Testing Targets on the Horizon

- ROS1 translocation
- MET amplification and mutations
- BRAF mutation
- PI3K mutations
- FGFR mutation

- TS expression levels
- KRAS mutations

- Other potential sources of DNA
  - Circulating tumour cells
  - Free plasma DNA
  - Urinary DNA
    - Reports of using urine to screen for T790M mutations in patients on TKIs presented at European Lung Cancer Conference 2015.

Next Generation Sequencing

- Offers multiplex testing
- We are currently validating Ion Torrent platform with the Oncomine chip.
- Differing sensitivities and specificities from current targeted testing
- Mutations and translocations can both be detected
- May allow testing of smaller samples but 'rubbish in rubbish out' still applies!
  - Important to not allow this to become an excuse for the submission of even smaller samples for diagnosis!

Issues

1. How do we improve reporting and testing practises across the UK?

Histopathology

Histopathology 2015, 67, 216–224. DOI: 10.1111/his.12638

Morphological and genetic classification of lung cancer: variation in practice and implications for tailored treatment

Paul Cane, Karen M Linklater,[1] Andrew G Nicholson,[2] Michael D Peake[3] & John Gosney[4]

Department of Histopathology, Guy's and St Thomas' NHS Foundation Trust, London, UK, [1]National Cancer Registration Service, London, UK, [2]Department of Histopathology, Royal Brompton and Harefield NHS Foundation Trust and National Heart and Lung Institute, Imperial College, London, UK, [3]Department of Respiratory Medicine, Glenfield Hospital, Leicester, UK, and [4]Department of Cellular Pathology, Royal Liverpool University Hospital, Liverpool, UK

Snapshot audit of reporting practices in 19 randomly selected hospital MDTs across England

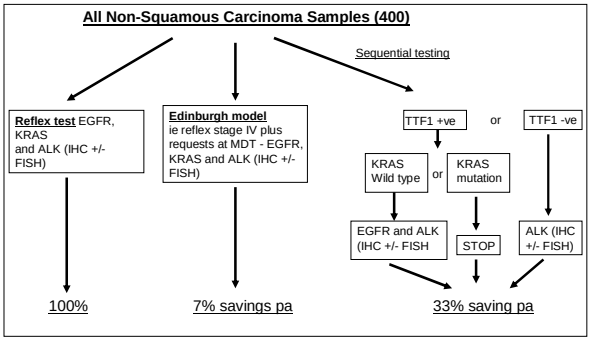
Table 3. Analysis for EGFR gene mutations

Centre	Instigation of analysis	Location of analysis	Type of tumour analysed	Number (%) of NSCLC analysed	Sensitizing mutations %
1	At MDT	In network	Adeno	49 (88)	20
2	At MDT	In network	Non-sq	24 (35)	0
3	At MDT	In network	Adeno	5 (23)	0
4	Oncologist	In network	Adeno	25 (50)	12
5	Pathologist	On site	Non-sq	55 (92)	7
6	At MDT	In network	Adeno	10 (50)	0
7	Pathologist	In network	Adeno	42 (70)	5
8	At MDT	Outside network	Non-sq	3 (12)	33
9	At MDT	In network	Adeno	16 (62)	13
10	At MDT	In network	Adeno	8 (35)	3
11	At MDT	In network	All NSCLC	27 (64)	0
12	At MDT	In network	Adeno	7 (64)	14
13	At MDT	Outside network	Adeno	17 (52)	18
14	At MDT	Outside network	Adeno	10 (53)	30
15	At MDT	Outside network	Non-sq	8 (47)	50
16	At MDT	Outside network	Adeno	5 (22)	20
17	Pathologist	Outside network	Non-sq	28 (62)	18
18	Pathologist	In network	Non-sq	7 (41)	29
19	At MDT	Outside network	Non-sq	12 (86)	0

Adeno, Adenocarcinoma; EGFR, Epidermal growth factor receptor; Non-sq, Non-squamous; MDT, Multidisciplinary team; NSCLC, non-small cell lung cancer.

Why is the range in % tested so wide 12 – 92%?
Why are some centres only testing adenocarcinomas and one centre testing all NSCLC (ie squamous carcinoma as well)?
Who or what should drive testing?

2. Is our current testing strategy pouring money down the drain?



K Walsh, Y Kheng, A Oniscu, DJ Harrison, WA Wallace.
Could molecular pathology testing in lung cancer be made more cost effective?
J Clin Path 2016 In Press

Summary

- The last 5 years has seen a revolution in the way lung carcinomas are reported and the tissue based investigations that can be carried out.
- Currently a huge amount of effort to detect small numbers of 'actionable cases' but this is likely to change as new targets come on stream
- Pathologists have a key role in this as we are the individuals with the ability to confirm the nature of the tissue obtained and steer appropriate investigations on often very limited material.
- We need to work closely with respiratory physicians, oncologists and clinical scientists to ensure optimal tissue preservation and appropriate testing.