



LUNG CYTOLOGY Predictive markers and molecular tests

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Pathologists' role

Historically	2016
<ul style="list-style-type: none"> Guiding the surgeons' hands Pattern recognition Tumour burden Basements (hiding behind a microscope) 	<ul style="list-style-type: none"> Oncologists' best mate Understanding disease Disease biology Integral part of the multidisciplinary team

Ancillary Studies in Cytology Challenges

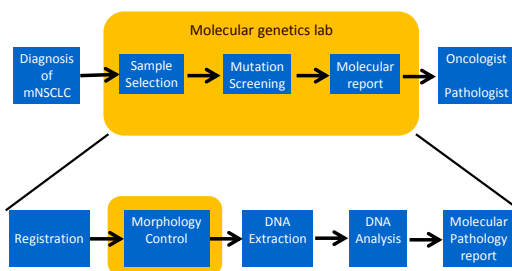
- To select the correct test for a limited sample quantity.
- Avoid jumping from a histological adapted technique directly to cytological material.
- Use appropriate controls for cytological material.

Pre-analytical issues

- The variability in material and fixatives is a major factor preventing standardization of some procedures using cytology.
- Spray and ethanol fixation results in better DNA quality than air drying (but both give reliable results in clinical specimens).
- Due the similarities with histological material, cell-blocks are more easy to handle on the molecular lab.

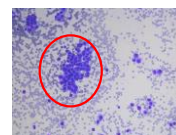
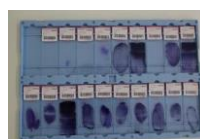
Dajmek A. Cancer Cytopathology 2013
Schmitt F. Cytopathology 2011

MOLECULAR TESTING WORKFLOW



Simple protocol for DNA extraction from archival stained FNA smears

- Slide Selection and Assessment**
For cases with a high tumor content (>20%) the marking of areas of tumors is unnecessary. (1 cell = 6 pg DNA)
- Removing the Coverslip**
48-72hs in xylene or substitute

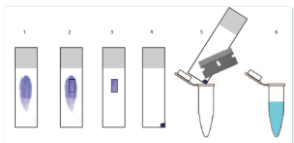


Enrichment by macrodissection if necessary.

Acta Cytologica 2011;50:1000-1001

Simple protocol for DNA extraction from archival stained FNA smears

3. Collecting the Tissue



4. Tissue Lysis and DNA Extraction

Acta Cytologica 2010;50(4):457

Minimizing Delays in DNA Retrieval: The "Freezer Method" for Glass Coverslip Removal.



. The slide is placed flat in the freezer (at a temperature of -20°C) for 1-3 minutes.

Santos GC et al. Cancer Cytopathology 2013

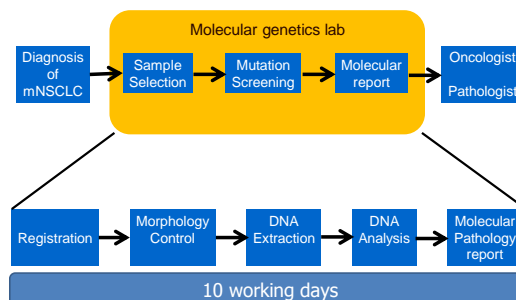
Schmitt FC. Cytopathology 2011

Rapid On-Site Evaluation

- The increasing need to assess specific targetable mutations and/or genetic aberrations implicates in a detailed evaluation of the collected material with formulation of a preliminary diagnosis followed by extra passes or adequate triage.
- Only an experienced cytopathologist can consistently and correctly decide on handling of the specimens and failure to do so, may result in direct negative consequences to patient care.

Cancer Cytopathology 2012

EGFR mutation status testing workflow



KEY POINTS TO USE MOLECULAR TECHNIQUES IN CYTOLOGY

- Collect good and well-preserved material.
- Validate molecular studies on cytological material.
- Control the cases morphologically.

THE PETHALS AND THORNS OF ROSE Disadvantages

- Need of an experienced on-site cytopathologist because relies only on morphology.
- Equivocal on-site diagnosis may prematurely end a procedure.
- Need of extra-time from the cytopathologist with financial under compensation of pathologist's time.

Cancer Cytopathology 2012

LUNG CYTOLOGY and TUMOR TYPING

PATHOLOGY CONSIDERATIONS FOR GOOD PRACTICE

- Small biopsy and cytology samples should be managed not only for diagnosis but also to maximize the amount of tissue available for molecular studies.

TABLE 3. Sensitivity and Specificity of Cytologic Tumor Subtyping

	Sensitivity, % (95% CI)	Specificity, % (95% CI)	PPV,* % (95% CI)	NPV, % (95% CI)	Accuracy, % (95% CI)
SCLC vs. non-SCLC	100 (29.2-100)	100 (97.9-100)	100 (97.9-100)	100 (97.9-100)	100 (98.1-100)
Squamous vs. nonsquamous	87 (66.4-97.2)	98 (94.6-99.6)	87 (66.4-97.2)	98 (94.6-99.6)	97 (93.2-98.6)
Adenocarcinoma vs. nonadenocarcinoma	98 (93.9-99.6)	79 (63.2-89.7)	94 (88.7-97.2)	92 (77.5-98.3)	93 (89.1-96.3)

- Cytology is a powerful tool in the diagnosis of lung cancer, in particular in the distinction of adenocarcinoma from squamous cell carcinoma.

J Thorac Oncol. 2011;6: 451-458

LUNG CYTOLOGY and TUMOR TYPING

TABLE 5. Frequency and Accuracy of Morphologic vs. Immunohistochemistry-Aided Diagnoses of Adenocarcinoma and Squamous Cell Carcinoma in Cytology

	Frequency	Accuracy
Diagnosis based on morphology	146 (88%)	96%
Diagnosis based on IHC	14 (9%)	100%
Diagnosis that needs IHC but cellularity insufficient (diagnosis: NSCLC-NOS)	5 (3%)	na

Ancillary Studies, Including Immunohistochemistry and Molecular Studies, in Lung Cytology

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Table 2 Common immunocytochemistry markers used in lung cytology

Lung Cancer Subtype	Markers				
	TTF-1	Napsin A	P63	P40	CK5/6 Neuroendocrine Markers
ADC	+/- *	+	-/+	-	-
SqCC	-	-	+	+	-
SCLC	+	-	-	-	+
NSCLC-NOS	+/- *	-	-	-	-
Metastatic carcinoma	-/+	-	-/+	-/+	-/+

* Especially mucinous ADC.

J Thorac Oncol. 2011;6: 451-458

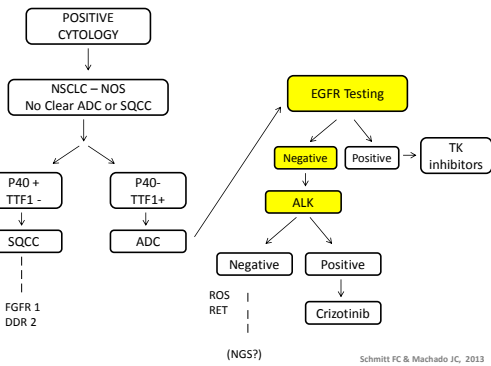
Review p40: A p63 Isoform Useful for Lung Cancer Diagnosis - A Review of the Physiological and Pathological Role of p63

Ana Rita Nogueira^{1,2}, Andre Albergaria^{1,2}, Fernando Schmitt^{1,2}

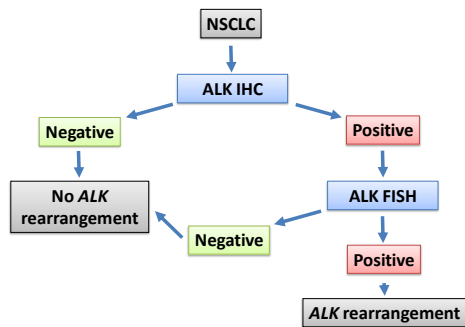
Table 1. Distribution of TTF-1, p63, and p40 in lung cancer

Histological diagnosis	ADC		SCLC		SE	SP	PPV	NPV	Rel.
	n	%	n	%					
TTF-1	115/120 (95)	95.8	0/1	0	0.97	1.00	1.00	0.99	95
p63	27/120 (22)	18.3	0/1	0	0.20	0.02	0.02	0.02	45
p40	20/120 (16)	13.3	0/1	0	0.16	0.01	0.01	0.01	33
TTF-1+p63	115/120 (95)	95.8	0/1	0	0.97	1.00	1.00	0.99	95
TTF-1+p40	115/120 (95)	95.8	0/1	0	0.97	1.00	1.00	0.99	95
TTF-1-p63	10/120 (8)	6.7	0/1	0	0.08	0.00	0.00	0.00	13
TTF-1-p40	10/120 (8)	6.7	0/1	0	0.08	0.00	0.00	0.00	13
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ALGORITHM FOR ANCILLARY TESTING IN NSCLC

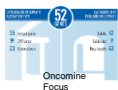


Diagnostic algorithm for ALK testing



3 tier approach to NGS in oncology

- Clinical**
 - Focused Panels detecting defined clinically relevant alterations (SNVs, CNVs, InDels, Fusion Transcripts)
- Translational**
 - Broad Panels detecting a large number of defined alterations covering the drug development (oncology pipeline) spectrum (recruitment for clinical trials)
- Exploratory**
 - Exome Sequencing (T/N)
 - RNAseq
 - WGS (T/N)



Genomic Analysis in Cytology

- Next generation sequence (NGS) has recently entered in the routine clinical molecular diagnostics by providing a highly multiplexed platform for simultaneous screening of multiple genes at a high analytical sensitivity with minimal amounts of DNA.
- Cytologic specimens offer a number of advantages in terms of molecular testing and minimally invasive procedures such as FNA are often used to establish a diagnosis.

Massive parallel sequencing to assess the mutational landscape of fine needle aspirate samples: a pilot study

Jose Luis Costa¹, Renel Gerhard¹, Esther Diana Rossi¹, Luis Cimes¹, Ana Justino¹, Jose Carlos Machado^{1,2}, **Fernando Schmitt^{1,3}**

Introduction
Next-generation sequencing (NGS) is allowing research in the field of oncology. The fast evolving technology is helping to move into the clinical diagnostic arena. The possibility of performing genomic studies in small amounts of material obtained for example, by fine needle aspiration (FNA), can minimize invasive procedures and allow the monitoring of cancer, including therapeutic response, with repeated testing. In this pilot study we aim to address the possibility of using this technology for assessing the mutational landscape of FNA samples.

Methods
Genomic DNA was isolated from four FNA samples collected in Thoracic Pleuroscopy Station. A single tube multiplex PCR amplification designed to detect 739 mutations from 46 oncogenes and tumor suppressor genes was performed using the Ion AmpliSeq Cancer Hotspot Panel assay. All amplicons were sequenced using the Ion Torrent PGM sequencer (Fig. 1).

Results & Discussion
Data from the PGM runs were processed using the Ion Torrent platform specific pipeline software Torrent Suite v3.2. The mutational landscape of the FNA samples was obtained using the Torrent Suite Variant Caller plugin v3.2.4 and the OncoPrint software (http://www.oncoprint.org/). The FNA samples were obtained from breast tumors (samples 1, 2 and 3) and in thyroid follicular tumor (sample 4) (Fig. 2). High quality genomic DNA was obtained from all samples. The multiplex PCR-based strategy resulted in the generation of 480 amplicons. This strategy allows multiplexing three samples in a chip 316 or running a single sample in a chip 314 (Fig. 2).

The use of two different bioinformatic tools for variant calling presented similar results (Table 2). Only one variant (p.S611P) in the gene IGF1R was differently called and confirmed to be a false-positive. The allele frequency of 11% of the alteration in IGF1R in the gene SMAD4 highlights the sensitivity of this technology. This variant was detected at a 2500x depth of coverage.

Variant Call	Variant
1	IGF1R p.S611P
2	IGF1R p.S611P
3	IGF1R p.S611P
4	IGF1R p.S611P

Conclusion
In this study we present a workflow that provides a simple genetic screening tool for FNA samples, in a fast and cost-efficient manner. This may provide a valuable tool for the management of cancer patients. This can be implemented in multiple laboratories and laboratories.

Abstract 354, USCAP 2013

Genomic Analysis in Cytology

- However, cytology specimens are still underutilized for molecular testing because most molecular testing requests are directed reflexively to the histology and cytology is used only when there is no concurrent core biopsy (CB) or the CB is insufficient for testing.
- The other issue is that molecular laboratories are not always validated all the different types of cytologic preparations (smears, cytopspins, LBC, cell-blocks) and prefer to treat all specimens as they do with the FFPE histology blocks.

Pre-analytical challenges for genomic analysis in cytology

- **Pre-analytical factors** are determinant for the success of NGS sequencing and recognizing these factors can enable better processing or triaging of specimens to further improve NGS success.
- In cytology specimens, these factors may be especially important because of the wide variations in sample types, preparations and adequacy criteria.

Chen H. et al. Cancer 2015

Pre-analytical factors that affects NGS analysis in cytology

- Specimen cellularity.
- Type of preparation.
- Type of fixative and stains.
- Type of glass slides.
- Tumour fraction.
- DNA yield
- Input DNA

Chen H. et al. Cancer 2015

Role of the Cytopathologists

- There is a wide interobserver variation among cytopathologists in estimating tumor fraction, even with predefined adequacy criteria.
- Some cytopathologists demonstrate high cancellation rates, resulting in low NGS failure rates, whereas others have low cancellation rates with high NGS failure rates.
- The variation among cytopathologists in tumor fraction estimation and selecting cases for NGS is also seen in the selection of substrate (cell blocks versus smears) and the number and type of glass slides sent for testing.

Roy-Chowdhuri et al. Cancer Cytopathology 2015

Consistency and Reproducibility of Next-Generation Sequencing and Other Multigene Mutational Assays: A Worldwide Ring Trial Study on Quantitative Cytological Molecular Reference Specimens

Umberto Malapelle, Clara Mayo-de Las Casas, Miguel A. Molina, Rafael Banell, Spasenija Savic, Michel Bibb, Lukas Bubendorf, Manuel Salto-Telles, Dario de Biasi, Giovanni Tallini, David H. Hwang, Lynette M. Sholl, Rajalakshmi Lathra, Sinehsa Roy-Chowdhuri, Birgit Weyand, Sara Vander Borgh, Edoardo Miasaglia, Massimo Bongiovanni, Daniel Stöber, Philippe Vieth, Fernando Schmitt, Alessandra Rizzo, Massimo Barberis, Francesco Pepe, Pasquale Trippola, Nicola Serra, Elena Vigliani, Claudio Bellizzi, Martin Fassun, Massimo Bogge, Carlos E. de Andrea, Maria D. Lucano, Fulvio Raspato, Gabriella Fontanini, Martina N. Nishiyama, Yari E. Nishiyama, Suzanne Kamel-Reid, Gilda da Cunha Santos, Giancarlo Troncone for the Molecular Cytopathology Meeting Group

This interlaboratory ring trial study shows that next-generation sequencing and other multigene mutational assays are robust and accurate with cytological samples. In particular, the performance of laboratories using next-generation sequencing is excellent, regardless of the platform or gene panel type.

Cancer Cytopathology 2017

EDITORIAL

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Molecular diagnostics and the training of future tissue- and cell-based pathologists

STAGE A 0-12 months	STAGE B 12-18 months	STAGE C 24-30 months	STAGE D 32 months
Introductory lectures on molecular diagnostics	Completion 2-3 month attachment in molecular diagnostics	Completion 2-3 month attachment in molecular diagnostics	Option 1: One year full-time tissue molecular diagnostics (see Table 2) Option 2: One year 'superspecialty' attachment with part-time practice in a subspecialty and part-time reporting the related molecular tests. Option 3: Mixture of diagnostics and research
Year 1 assessment at end of year to next stage	Exit stage with FREC/PA Part 1 examination	Exit stage with FREC/PA Part 2 examination with/without modules in anatomy and generalist cytology (additional 3 months each)	End of training

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