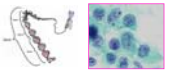
Dr. Robert Y. Osamura

Director, Department of Diagnostic Pathology, SANNO Hospital,
International University of Health and Welfare (IUHW) Graduate School,
Japan

Ancillary and Molecular Testing

Learning Objectives:



1. Principles of molecular techniques
2. Applications of molecular techniques in cancer cytopathology
    a. In situ hybridization
    b. Mutational analysis
3. Cytological materials for molecular testing

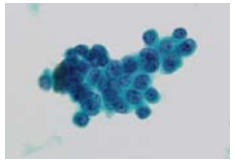
What is genomic(molecular) pathology in cancers?

Analysis of changes of genes of the cancer cells.
- Amplification
- Mutation point mutation deletion mutation
- Rearrangement

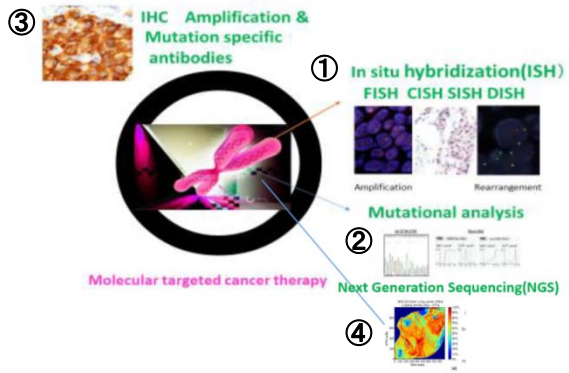
To predict response to the molecular targeted therapy

In situ hybridization

Direct sequencing
Allele specific detection



Four ways to detect genetic changes with NGS



Genomic(molecular) pathology & Cytology

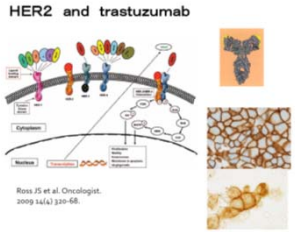
Immunohistochemistry
- Over-expression:HER2
- Mutation
  - Positive staining:p53
  - Negative staining:DAXX/ATRX
  - Mutation specific antibody:EGFR BRAF KRAS
- Re-arrangement:ALK

Gene expression and changes
- Amplification:In situ hybridization(ISH) FISH CISH DISH
- Rearrangement & translocation:FISH CISH DISH
- Mutation point mutation deletion insertion
  - Sanger direct sequencing Allele specific detetion

Next Generation Sequencing(NGS)

Application of Cytological materials to HER2 FISH,CISH,SISH DISH

Direct smear(FNA)
Fresh Archival
Liquid based cytology(LBC)
Cell block

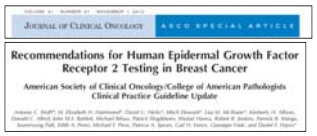


HER2 testing 2013

1.Immunohistochemistry 3+ >10%
2.Single probe ISH <4 4-6 >6
3.Dual probe ISH >2

HER2 testing 2007
"Equivocal→ Retest
IHC 3+ >30%
FISH 1.8-2.2

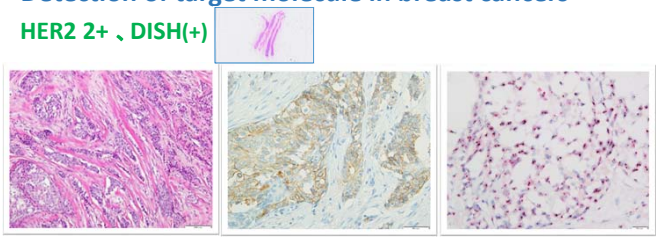
Algorithm



(Arch Pathol Lab Med. doi: 10.5858/arpa.2013-0953-SA)

Detection of target molecule in breast cancers

HER2 2+ 、DISH(+)

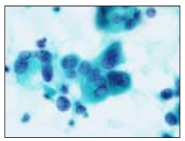


Invasive ductal carcinoma | HER2 2+ | DISH(+) HER2/Chr17 ratio 3.84

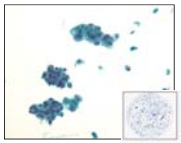
HER2 positive → Anti-HER2 therapy

Small specimens utilized for molecular(genomic) analysis

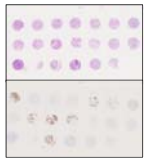
CNB



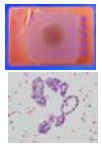
FNA



LBC



Tissue Microarray(TMA)

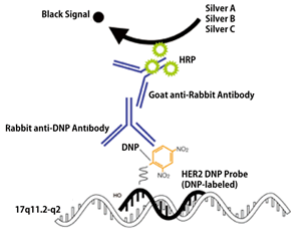


Cell block

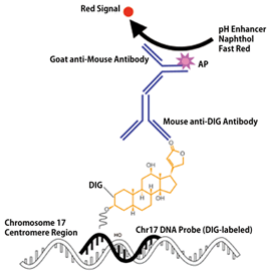
Laser capture microdissection(LCM)

Dual Color *in situ* Hybridization(DISH)

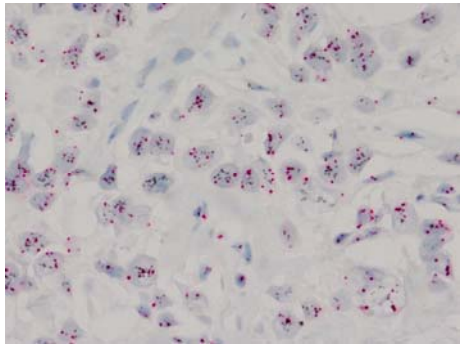
Step1
Silver ISH for HER2



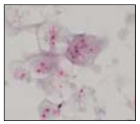
Step2
RED ISH for Chromosome 17



HER2 DISH on histology and cytology Invasive ductal carcinoma



FNA DISH



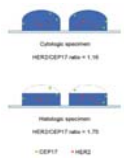
Histology 2.9 HER2 amplification Cytology 2.7

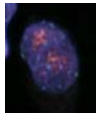
Molecular(Genomic) pathology: Cytology vs Histology

Cytology: FNA EUS-FNA LBC Cell block
Histology:Formalin-fixed paraffin embedded(FFPE) sections

Cytology:Whole nuclei ideal
Histology:Sectioned nuclei
Numbers of genes may differ
HER2/Ch17 ratio:lower in histology

Cytology:No paraffin embeddig
Histology:Paraffin embedding
DNA RNA preservation

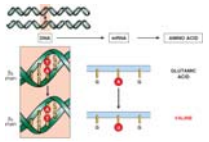




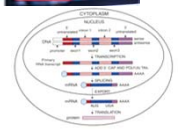
Red:HER2 Green:Ch17
HER2/Ch17
Ratio higher in histology

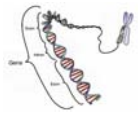
Cancer. 2008 Apr 25;114(2):134-40..

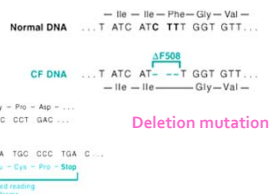
Gene Mutations



Point mutation







Methods to detect mutations

Sanger's Direct sequencing
Pyrosequencing
Allele specific analysis
idensy® (Arkray)

Next generation sequencing
Whole genome sequencing(NGS)
Targeted NGS

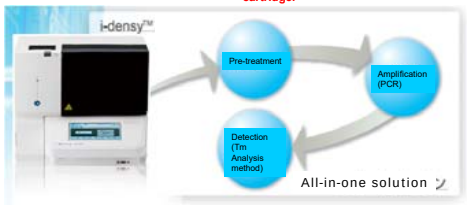
Allele specific mutational detection

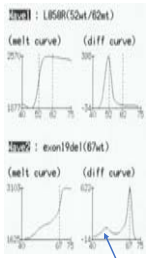
Japanese word for gene→ idenshi 遺伝子→ i-densy

i-densy website
http://i-densy.arkray.co.jp/index.html

Fully automated gene analyzer
i-densy™ IS-5320

- ✓ 80 min from start to result output and using whole blood
- ✓ Measurement of up to 3 SNPs with one cartridge.





EGFR Deletion mutation exon 19

EGFR in lung caner

Point mutation on exon 21 L858R
Deletion mutation exon 19 746-750

Point mutation on exon 21
more responsive to gefitinib Sasaki et al. Clin Cancer Res 11:2924, 2006
L858R mutation good response Sone et al. Lung cancer 54:103, 2006

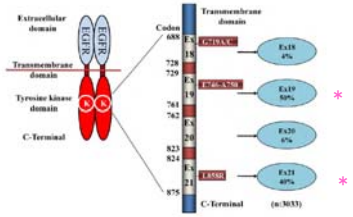
Exon 19 deletion Longer overall survival
Higher response rate
Longer TTP

KRAS mutation (40%) No response to gefitinib Kosaka et al.2004
EGFR immunohistochemistry- not useful

EGFR FISH amplification No higher response No longer TTP No longer OS
Mutation, not amplification, may serve as predictors of gefitinib response
Sone et al. Cancer 109:1836, 2007

Applications of the mutational analysis on tissues(histology) and cells(cytology)

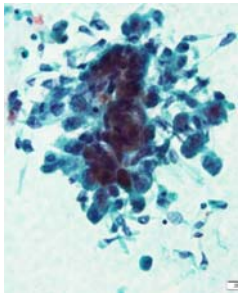
EGFR and lung cancers



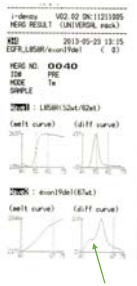
* 90%

Yoshida T et al. Biochem Pharmacol. 2010 May 25. Frequency of EGFR mutations in NSCLC (n = 3033)

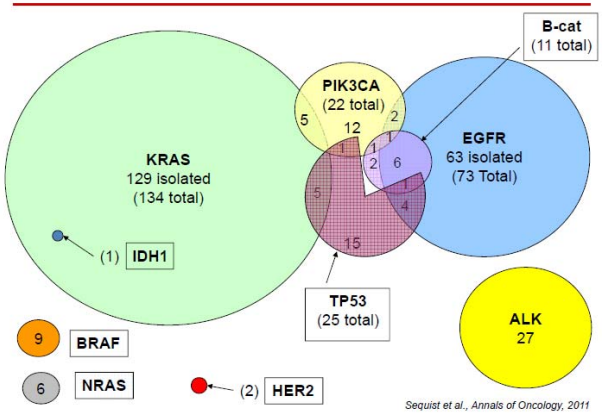
Mutational anaysis from cytology specimens EGFR exon 19 deletion



i-densy® Results



Overlap of Mutations - NSCLC



Sequist et al., Annals of Oncology, 2011

Cytology and ALK FISH

Cytology Specimens Offer an Effective Alternative to Formalin-Fixed Tissue as Demonstrated by Novel Automated Detection for ALK Break-Apart FISH Testing and Immunohistochemistry in Lung Adenocarcinoma

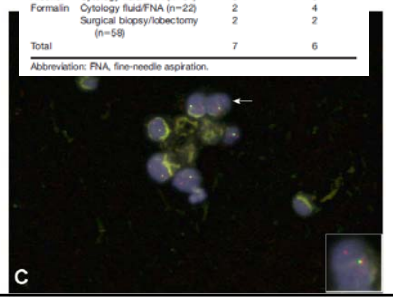
Priska Rosenkilde, MD[1], Lloyd M. Hutchinson, PhD[2], Joann Garver, BS[2], Bruce Wintle, MD[2], Edie Cowan, MD[2], and Elizabeth M. Hunter, MD[2]

Cancer (Cancer Cytopathol) 2014;000:000–000.

TABLE 3. Technical Failure by Fixation and Specimen Type

Fixation	Specimen Type	Insufficient Cells	No Hybridization Signals
Alcohol	Cytology fluid/FNA (n=34)	3	0
Formalin	Cytology fluid/FNA (n=22)	2	4
	Surgical biopsy/lobectomy (n=58)	2	2
Total		7	6

Abbreviation: FNA, fine-needle aspiration.



EGFR mutation-specific antibodies

Jian Yu et al. Mutation-Specific Antibodies for the Detection of EGFR Mutations in Non -Small-Cell Lung Cancer Clin Cancer Res. 2009 May 1;15(9):3023-8.

Activating mutations within the tyrosine kinase domain of epidermal growth factor receptor (EGFR) are found in approximately10% to 20% of non - small-cell lung cancer (NSCLC)patients and are associated with response to EGFR inhibitors.

The most common NSCLCassociated EGFR mutations are **deletions in exon 19 and L858R mutation in exon 21,** together accounting for9 0% of EGFR mutations. Home made antibodies

Screening a panel of 340paraffin-embedded NSCLC tumor samples with these antibodies showed that the **sensitivity of the immunohistochemistry assay is 92%, with a specificity of 99%** as compared with direct and mass spectrometry - based DNA sequencing.

This simple assay ford etection of EGFR mutations in diagnostic human tissues provides a rapid, sensitive, specific, and cost-effective method to identify lung cancer patients responsive to EGFR-based therapies

Next Generation Sequencing(NGS)

Detection of multiple mutations of the multiple target cancer genes
Detection of druggable genes and the genes for therapeutic prediction

Challenges and opportunities of next-generation sequencing: a cytopathologist's perspective

E. Vigliar, U. Malapelle, C. de Luca, C. Bellevicine and G. Troncone
*Pathology Division, Department of Public Health, University of Naples Federico II, Naples, Italy

Cytopathology 2015, **26**, 271–283

able 1. Head-to-head comparison of on Torrent/PGM and Illumina/Mi eq technologies

Sequencing platforms	Ion Torrent/PGM	Illumina/Mi Seq
Principle	pH change monitoring	Reversible dye terminators
Amplification	Emulsion PCR	Bridge PCR
Read length	Up to 250 bp	Up to 250 bp
Principal error type	Homopolymeric regions	Substitution
DNA input (ng)	10	30–270
Number of neoplastic cells	100–1000	5000–15000
Run time (h)	4	27

FNA Smears as a Potential Source of DNA for Targeted Next-Generation Sequencing of Lung Adenocarcinomas

Amanda L. Treece, MD[1]; Nathan D. Montgomery, MD, PhD[2]; Nirali M. Patel, MD[2,3]; Chris J. Civalier, BS[2]; Leslie G. Dodd, MD[2]; Margaret L. Gulley, MD, PhD[2,3]; Jessica K. Booker, PhD[2]; and Karen E. Weck, MD, PhD[2,3]

Cancer Cytopathology Month 2016

TABLE 3. Sequencing Success by Clinical Specimen Type

Specimen Type	Successful Cases, No.	Total Cases, No.	Successful, %
FNA direct smears	12	12	100[a]
Surgical resections	55	57	96[b,c]
Body fluid cell blocks	9	10	90
Small biopsies	48	60	80[b]
FNA cell blocks	23	33	70[a,c]

Abbreviation: FNA, fine-needle aspiration.
[a] Statistically significant difference between FNA direct smears and FNA cell blocks ($P = .03$).
[b] Statistically significant difference between surgical resections and small biopsies ($P = .0004$).
[c] Statistically significant difference between surgical resections and FNA cell blocks ($P = .0003$).

A validation set of 10 slides from 9 patients with prior clinical epidermal growth factor receptor (*EGFR*) Sanger sequencing and *KRAS* pyrosequencing (5 *KRAS*-positive/*EGFR*-negative and 4 *KRAS*-negative/*EGFR*-negative) underwent DNA extraction, quality assessment, and targeted NGS. Subsequently, lung adenocarcinoma specimens submitted for NGS solid tumor mutation panel testing in 1 calendar year (60 biopsies, 57 resections, 33 FNA cell blocks, 12 FNA smears, and 10 body fluid cell blocks) were reviewed for specimen adequacy, sequencing success, and DNA quality. **RESULTS:** All 10 validation

according to ΔC, was significantly better with FNA smears versus biopsies, resections, and FNA cell blocks. **CONCLUSIONS:** FNA smears of lung adenocarcinomas are high-quality alternative specimens for a targeted NGS panel with a high success rate in clinical practice. *Cancer (Cancer Cytopathol)* 2016;000:000–000. © 2016 American Cancer Society.

Methods in Pathology

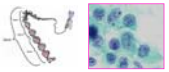
Next-generation sequencing-based multi-gene mutation profiling of solid tumors using fine needle aspiration samples: promises and challenges for routine clinical diagnostics

Rashmi Kanagal-Shamanna, Bryce P Portier, Rajesh R Singh, Mark J Routbort, Kenneth D Aldape, Brian A Handal, Hamed Rahimi, Neelima G Reddy, Bedia A Barkoh, Bal M Mishra, Abhaya V Paladugu, Jawad H Manekia, Neda Kalbor, Sinchita Roy Chowdhuri, Gregg A Staerkel, L Jeffrey Medeiros, Rajyalakshmi Luthra and Keyur P Patel

Department of Hematopathology, The University of Texas M.D. Anderson Cancer Center, Houston, TX, USA

46 cancer-related genes using a 318-chip on Ion PGM Sequencer. All tested samples underwent successful targeted sequencing of 46 genes. We showed 100% concordance of results between next-generation sequencing and conventional test platforms for all previously known point mutations that included ***BRAF***, ***EGFR***, ***KRAS***, ***MET***, ***NRAS***, ***PIK3CA***, ***RET*** and ***TP53***, deletions of ***EGFR*** and wild-type calls. Furthermore, next-generation sequencing detected variants in 19 of the 31 (61%) patient samples that were not detected by traditional platforms, thus increasing the utility of mutation analysis; these variants involved the ***APC***, ***ATM***, ***CDKN2A***, ***CTNNB1***, ***FGFR2***, ***FLT3***, ***KDR***, ***KIT***, ***KRAS***, ***MLH1***, ***NRAS***, ***PIK3CA***, ***SMAD4***, ***STK11*** and ***TP53*** genes. The results of this study show that next-generation sequencing-based mutational profiling can be performed on fine needle aspiration cytological smears and cell blocks. Next-generation sequencing can be performed with only nanograms of DNA and has better sensitivity than traditional sequencing platforms. Use of next-generation sequencing also enhances the power of fine needle aspiration by providing gene mutation results that can direct personalized cancer therapy.
Modern Pathology (2014) **27**, 314–327; doi:10.1038/modpathol.2013.122; published online 2 August 2013

Ancillary and Molecular Testing



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   a. In situ hybridization
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3. Cytological materials for molecular testing

References:

Osamura RY. Roles of pathologists in molecular targeted c ancer therapy. J Cell Mol Med.2009 Nov-Dec;13(11-12):4286-90

Kato N, Itoh H, Serizawa A, Hatanaka Y, Umem ura S, Osamura RY.Evaluation of HER2 gen e amplification in invasive breast cancer using a dual-color chromogenic in situ hybridization (dual CISH).Pathol Int. 2010 Jul;60(7):510-5.

Itoh H, Miyajima Y, Umemura S, Osamura RY.Lower HER-2/chromosome enumeration probe 17 ratio in cytologic HER-2 fluorescence in situ hybridization for breast cancers: three-dimensional analysis of intranuclear localization of centromere 17 and HER-2 signals.Cancer. 2008 Apr 25;114(2):134-40.

Kanagal-Shamanna R, Portier BP, Singh RR,et al. Next-generation sequencing-based multi-gene mutation profiling of solid tumors using fine needle aspiration samples: promises and challenges for routine clinical diagnostics.Mod Pathol. **2014** Feb;27(2):314-27.

Treece AL, Montgomery ND, Patel NM et al. FNA smears as a potential source of DNA for targeted next-generation sequencing of lung adenocarcinomas. Cancer Cytopathol. 2016 Jun;124(6):406-14.