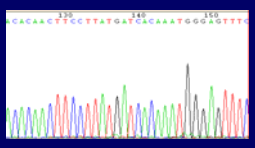
EGFR and K-RAS mutation analysis

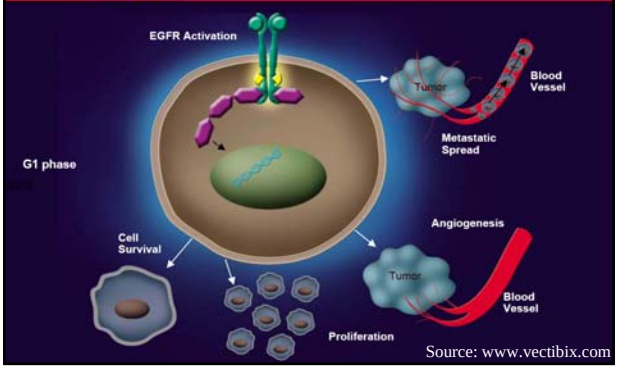


Daniëlle A.M. Heideman, PhD

Department of Pathology, VUmc

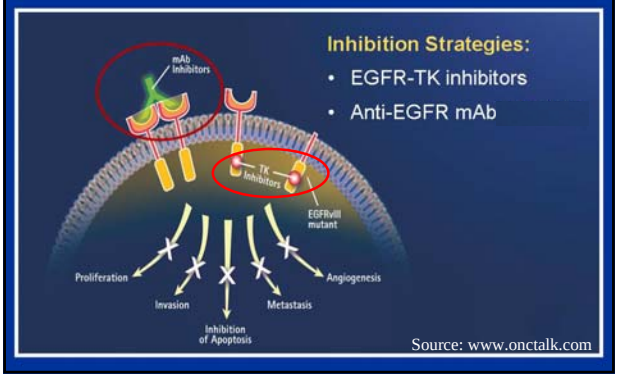
dam.heideman@vumc.nl

EGFR Activation Enhances Pathways Important for Tumor Cell Growth



Source: www.vectibix.com

Mechanisms of Inhibiting the EGFR Axis

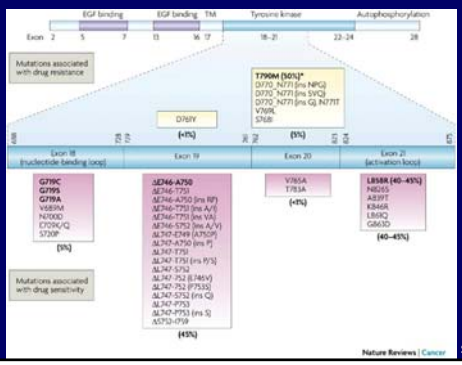


Source: www.onctalk.com

Prediction of responsiveness and efficacy of EGFR-targeted therapies (moAb or TKIs)

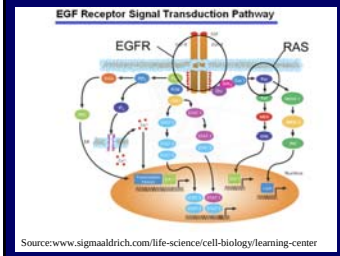
-> Increasing evidence points to the mutational status of EGFR and K-ras genes as effective molecular predictors

EGFR

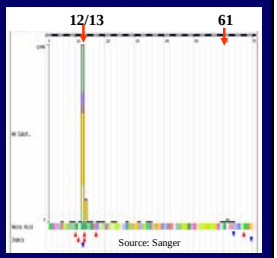


Sharma et al. 2007

Relevance of K-ras activating mutations for EGFR-directed therapies



Source:www.sigmaaldrich.com/life-science/cell-biology/learning-center



Source: Sanger

As a consequence, mutational screening tests for K-ras and EGFR may provide a direct and valuable guidance for clinicians to make decision on EGFR-targeted therapies.

Current indications (January 2010; may change in time)

1) EGFR x19/x21 -> EGFR-TKI lung cancer
2) K-ras -> contra anti-EGFR moAb colorectal cancer

Mutational analysis

Requirements for use in clinical / diagnostic setting include, amongst others:

- validated assay

- sufficient sensitivity
  limiting factors:
  - paraffin material (cross-linked and/or fragmented DNA)
  - tumor cell % -> enrichment by macro/micro-dissection
  - limiting amount of material (biopsies/cytology)

- high-throughput / non time-consuming

- cost-effective

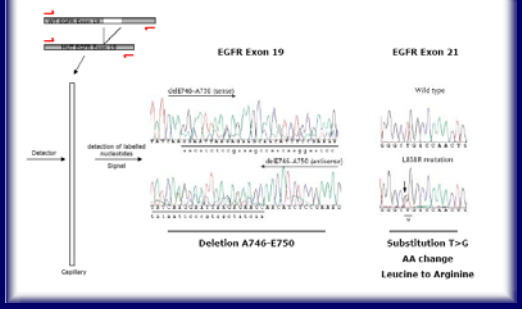
Variety of assays

In-house assays and commercial assays

- (nested-) PCR following cycle sequencing
- fragment analysis / RFLP
- SNaPshot primer extension assay
- point-EXACCT
- real time PCR
- melting analysis
- PCR followed by strip hybridisation

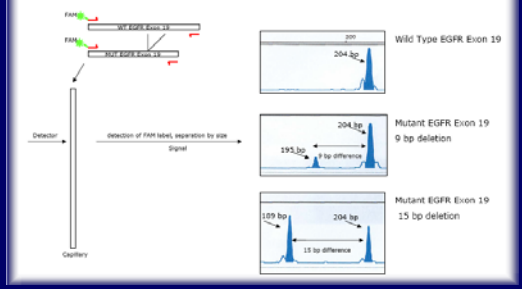
Some of which will be discussed below.

PCR and direct sequencing



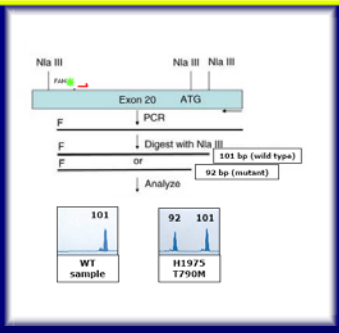
Courtesy of M. Gallegos-Ruiz

Separation by size
EGFR exon 19 deletions

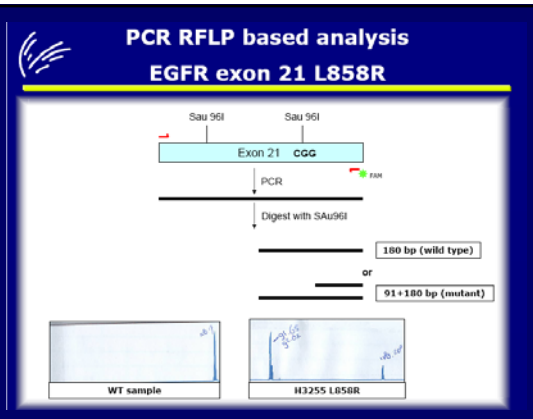


Courtesy of M. Gallegos-Ruiz

PCR RFLP based analysis
EGFR exon 20 T790M



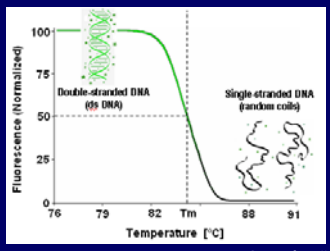
Courtesy of M. Gallegos-Ruiz



Courtesy of M. Gallegos-Ruiz

High Resolution Melting (HRM)

Principle: PCR with inclusion of an intercalating fluorescent dye, with subsequent melting curve analysis.



Source: http://www.dna.utah.edu/Hi-Res

High Resolution Melting (HRM)

The melting curve of a PCR product depends on o.a.
- GC content
- length
- sequence
- heterozygosity

-> Different genetic sequences melt at slightly different rates

Comparing the melting curve of a specimen to a reference (wild type) allows scanning for any sequence variation

-> Pre-screen assay with possibility to sequence PCR products to confirm genotype

HRM EGFR/K-ras panel

Panel of HRM assays to prescreen for mutations:

K-ras x1	-> a.o. codon 12/13
K-ras x2	-> a.o. codon 61
EGFR x19	-> a.o. deletions
EGFR x20p	-> a.o. ins 770/771
EGFR x20d	-> a.o. T790M
EGFR x21	-> a.o. L858R

Kramer et al. 2009
Heideman et al. 2009

Procedure of mutation detection by HRM

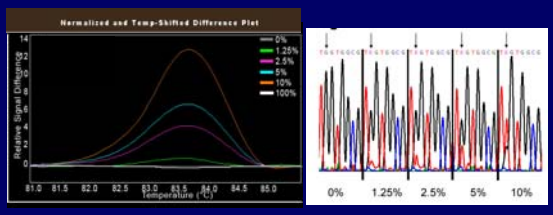
1) run cycling and melting program

2) check for correct amplification curves

3) check for melting curves and melting peaks (amplimer area and probe area, if applicable)

4) evaluate normalized, temperature-shifted difference plot (amplimer)

Analytical performance: High analytical sensitivity of HRM and subsequent sequencing



example: K-ras exon 1 (G12C)
2.5-5% mutant DNA in a background of wt can be well discriminated from background/noise signal

Kramer et al. Cell Oncol 2009

Performance of HRM on DNA isolated from FFPE-tissue in comparison to conventional nested-PCR/sequencing

1. High genotype agreement of HRM following sequencing with conventional nested-PCR following cycle-sequencing (kappa > 0.95)
2. Less-test failures
3. More mutations detected by HRM (likely related to the higher sensitivity as compared to conventional assay)

Kramer et al. 2009
Heideman et al. 2009

K-ras: high genotyping agreement of HRM with conventional cycle sequencing assay

Comparison of genotype findings of HRM and nested-PCR assays.

HRM \ nested-PCR	WT	G12A	G12C	G12D	G12S	G12V	G13C	G13D	NTD	
WT	49								6	55
G12A	1	5								6
G12C			10						1	11
G12D	1			6					1	8
G12S					1					1
G12V						7				7
G13C							1		1	2
G13D								2	1	3
NTD									5	5
total	51	5	10	6	1	7	1	2	15	98

NTD, not to determine;

Overall agreement in genotyping: kappa value of 0.96.

Kramer et al. 2009

EGFR: high genotyping agreement
of HRM with conventional cycle sequencing assay

EGFR genotype findings of HRM following sequencing in relation to findings of nested-PCR following sequencing

Nested-PCR	WT	Deletion exon 19	Deletion exon 19 + T790M	Insertion exon 20	R836R	P848L	L858R	NTD	Total
HRM									
WT	22							1	23
Deletion exon 19		15							15
Deletion exon 19 + T790M		1	3						4
Insertion exon 20				5					5
R836R					4				4
P848L						4			4
L858R	1						9		10
NTD								3	3
Total	23	16	3	5	4	4	9	4	68

Notes: NTD = not to determine;

Heideman et al. 2009

Conclusion

Increased interest in mutation analysis in MD in PA

Current indications (January 2010; may change in time)
1) EGFR x19/x21 -> EGFR-TKI lung cancer
2) K-ras -> contra anti-EGFR moAb colorectal cancer

Novel indications likely (b-raf, PIK3CA, ...)

Variety of assays available

Molecular assay useful in clinical/ diagnostic setting
- applicable to routine material (FFPE / cytology)
- sensitive: low-abundant mutants detectable
- fast and accurate
- prescreen technique -> high throughput
- allows for genotyping

Acknowledgements

Departments of Pathology & Medical Oncology & Pulmonary Diseases

Special thanks to the technicians of the Molecular Pathology Unit, Histo/Immuno Unit and Cytology Unit

Contact:
dam.heideman@vumc.nl