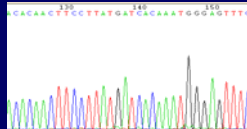
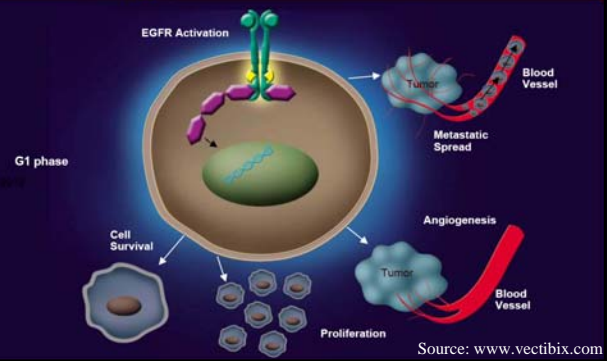


EGFR and K-RAS mutation analysis

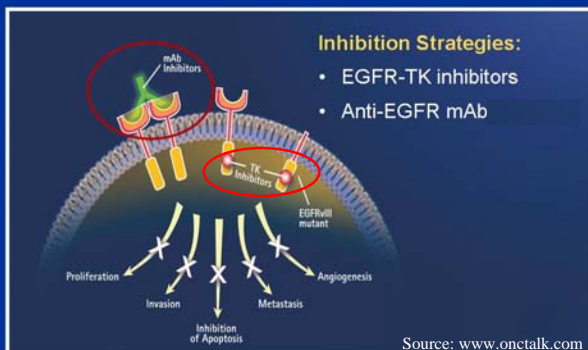


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EGFR Activation Enhances Pathways Important for Tumor Cell Growth



Mechanisms of Inhibiting the EGFR Axis



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

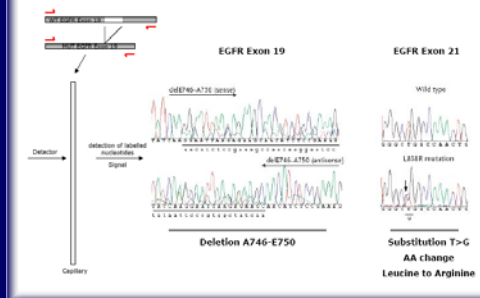
Variety of assays

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

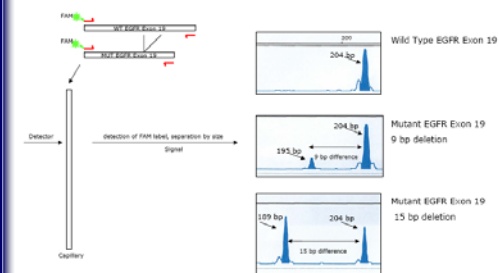
Some of which will be discussed below.

PCR and direct sequencing



Courtesy of M. Gallegos-Ruiz

Separation by size EGFR exon 19 deletions

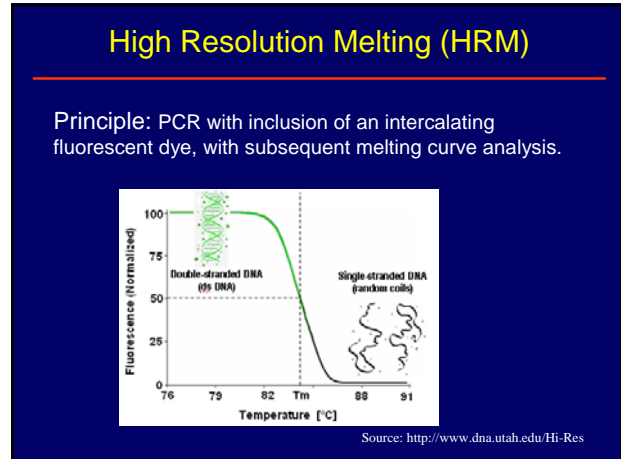
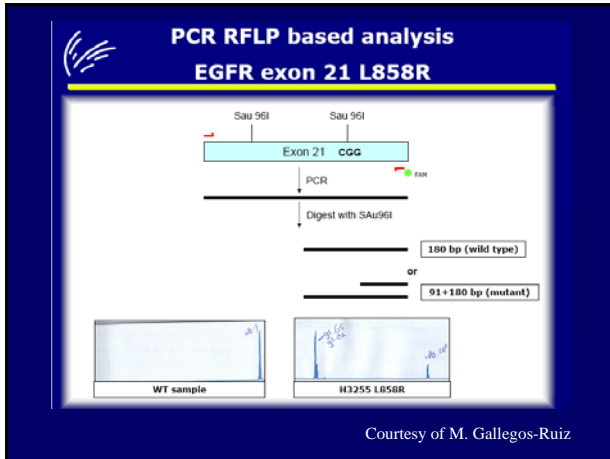


Courtesy of M. Gallegos-Ruiz

PCR RFLP based analysis EGFR exon 20 T790M



Courtesy of M. Gallegos-Ruiz



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:

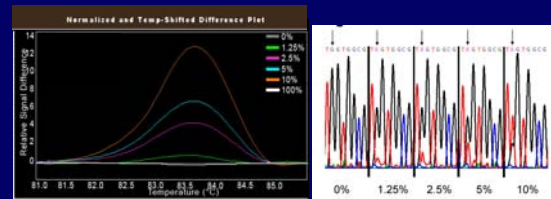
K-ras x1	-> a.o. codon 12/13
K-ras x2	-> a.o. codon 61
EGFR x19	-> a.o. deletions
EGFR x20p	-> a.o. ins 770/771
EGFR x20d	-> a.o. T790M
EGFR x21	-> a.o. L858R

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.

nested-PCR	WT	G12A	G12C	G12D	G12S	G12V	G13C	G13D	NFD
HRM									
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
NTD									5
total	51	5	10	6	1	7	1	2	15

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

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