Predictive Molecular Testing: What are the New Tools?

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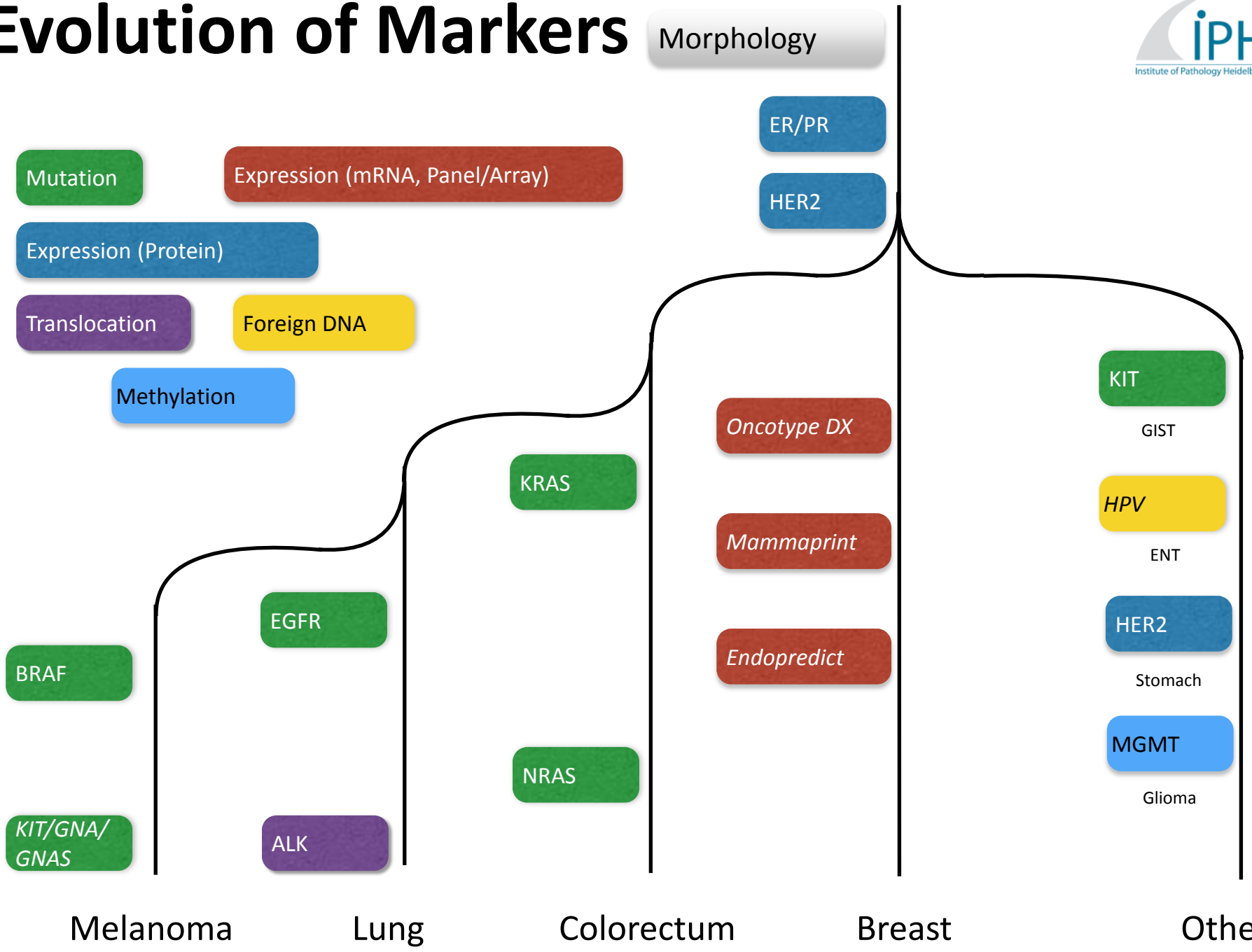
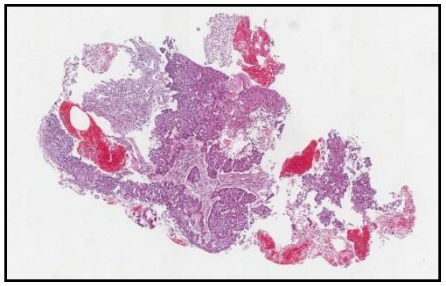
Topics

- Panel Sequencing
- ‚Liquid Biopsy‘
- Image Analysis
- Umbrella Concepts

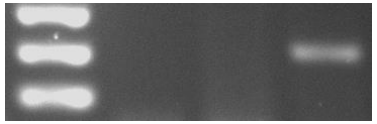
Evolution of Markers



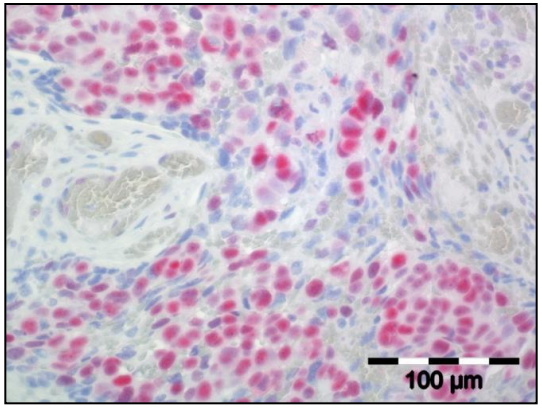
Complexity of Technologies



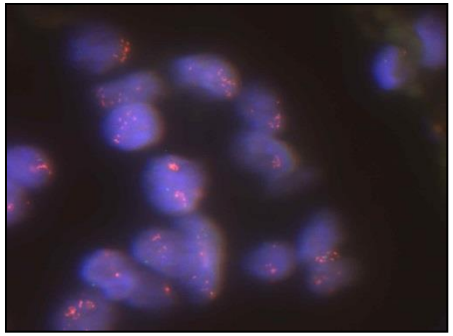
Histo



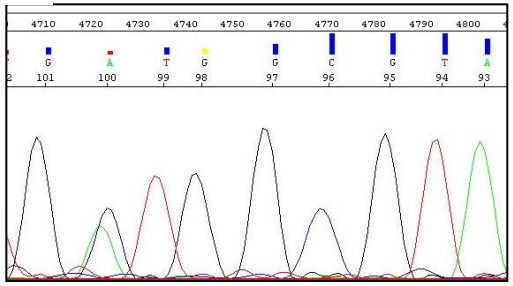
PCR-
Technologies



IHC

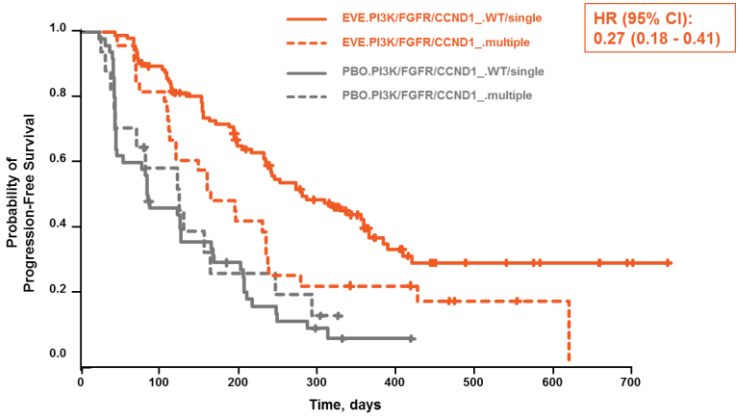


FISH, CISH



Sequencing (Sanger,
Pyro, NG)

New Single Markers

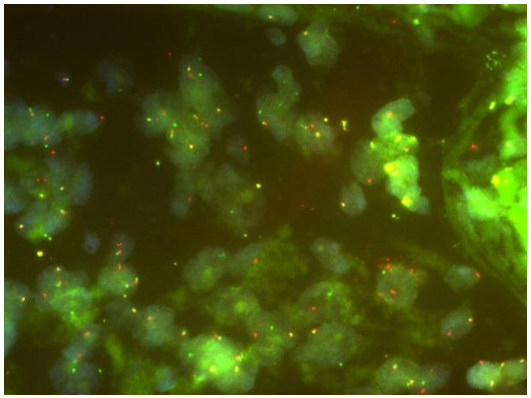


Established marker – new entity

e.g.. BRAF mutation in lung

Established marker – new drug

e.g. RAS mutation for MEK-inhibitor in CRC, lung, melanoma



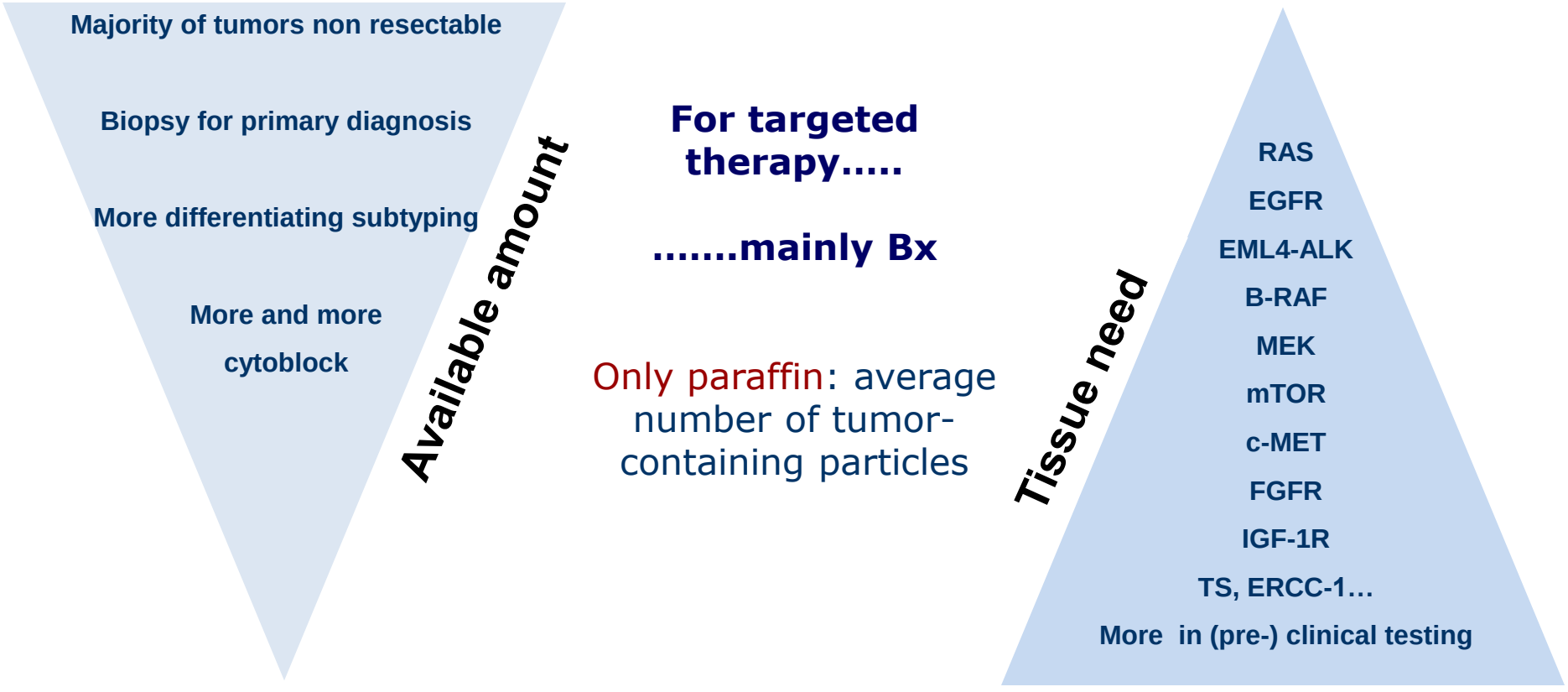
New marker – established drug

e.g. PIK3CA-mutation for mTOR-inhibitor in breast;
KRAS-RAS extension in CRC

New marker – new drug

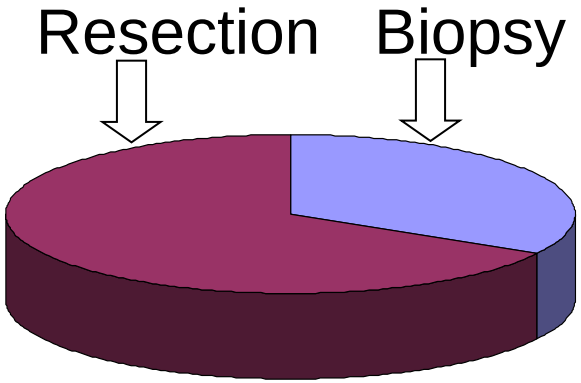
e.g. Met-expression for Met-inhibitor in stomach

Developments of Biopsy Diagnostics

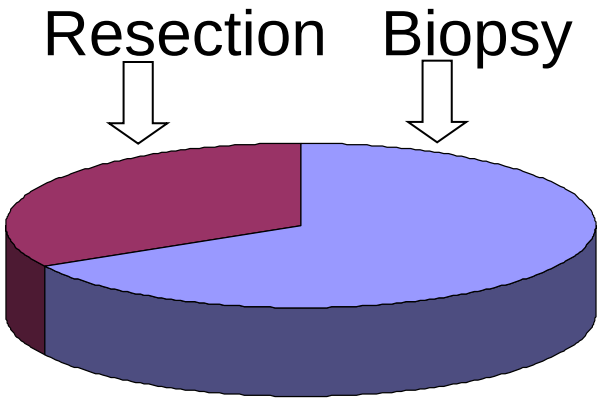


Number of sections extremely variable and depending on:
Experience of endoscopist, instrumentation
thickness of sections, number of procedures , temperature,
Experience of TA, experience of pathologists, algorithms etc.

The Biopsy Challenge



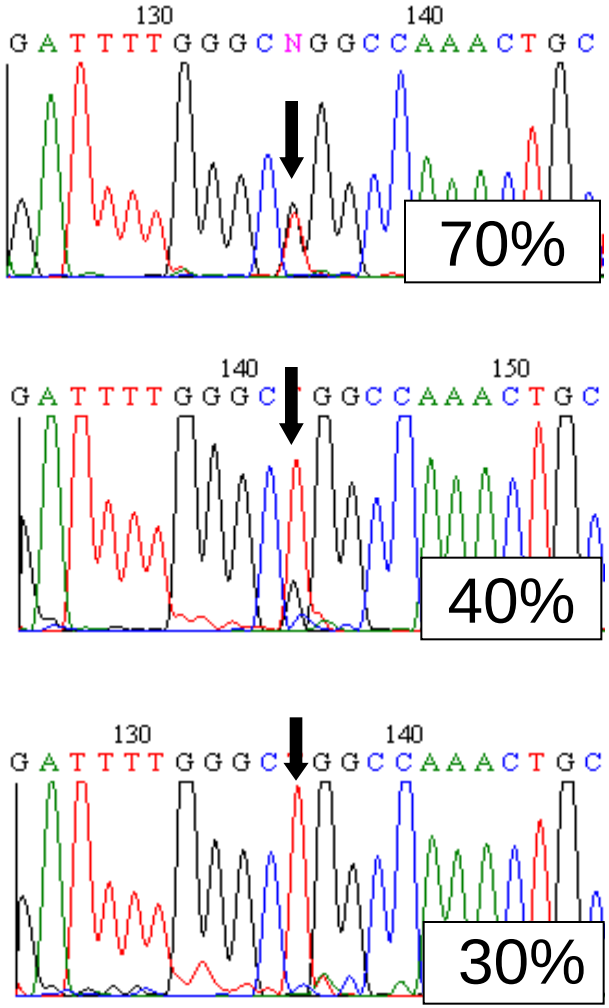
2009

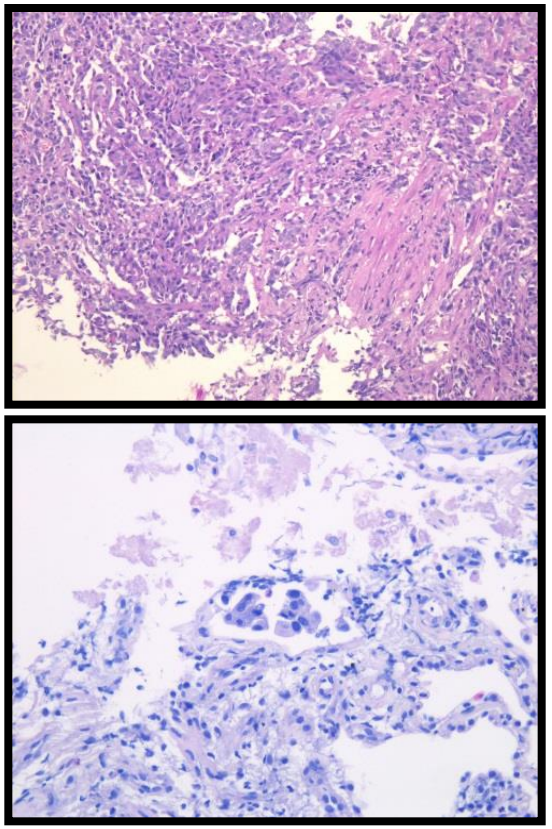


1/2010

- **Time pressure (patient management)**
- **Extreme increase case numbers**
- **Critical amount of material**
- **Critical tumor content**
- **Microdisscktion**

Tumor Cell Concentration





Critical amount:
Tumor cell content >40%
(20% mutated allele)

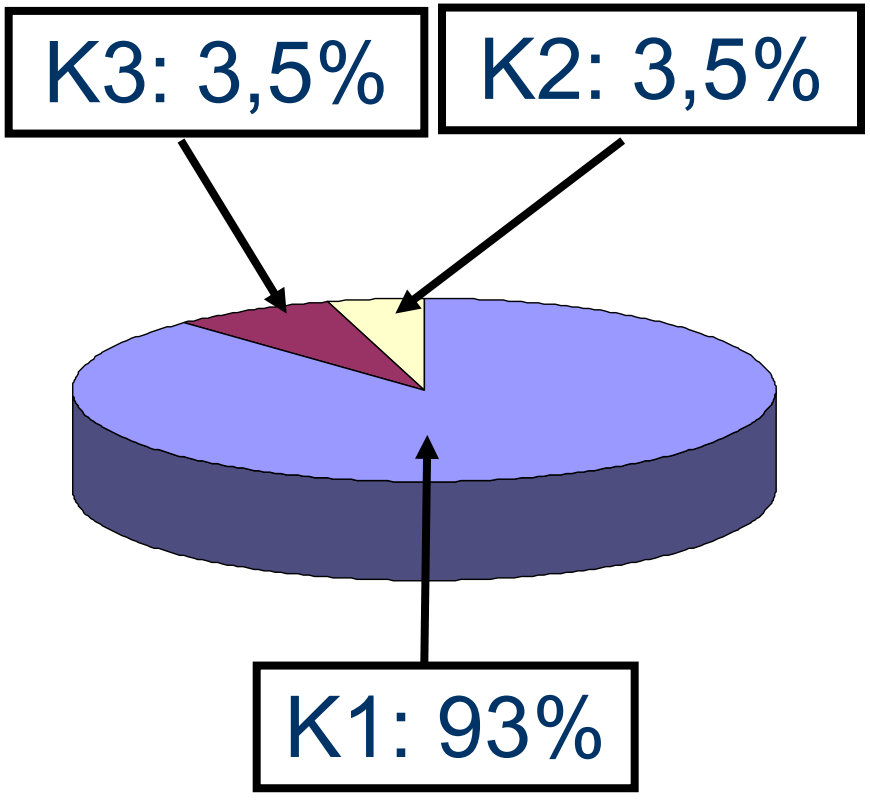
Warth et al., Virchows Arch (2012)

Reporting/Drop Out

Category 1:
Sufficient tumor, high tumor content, no restriction

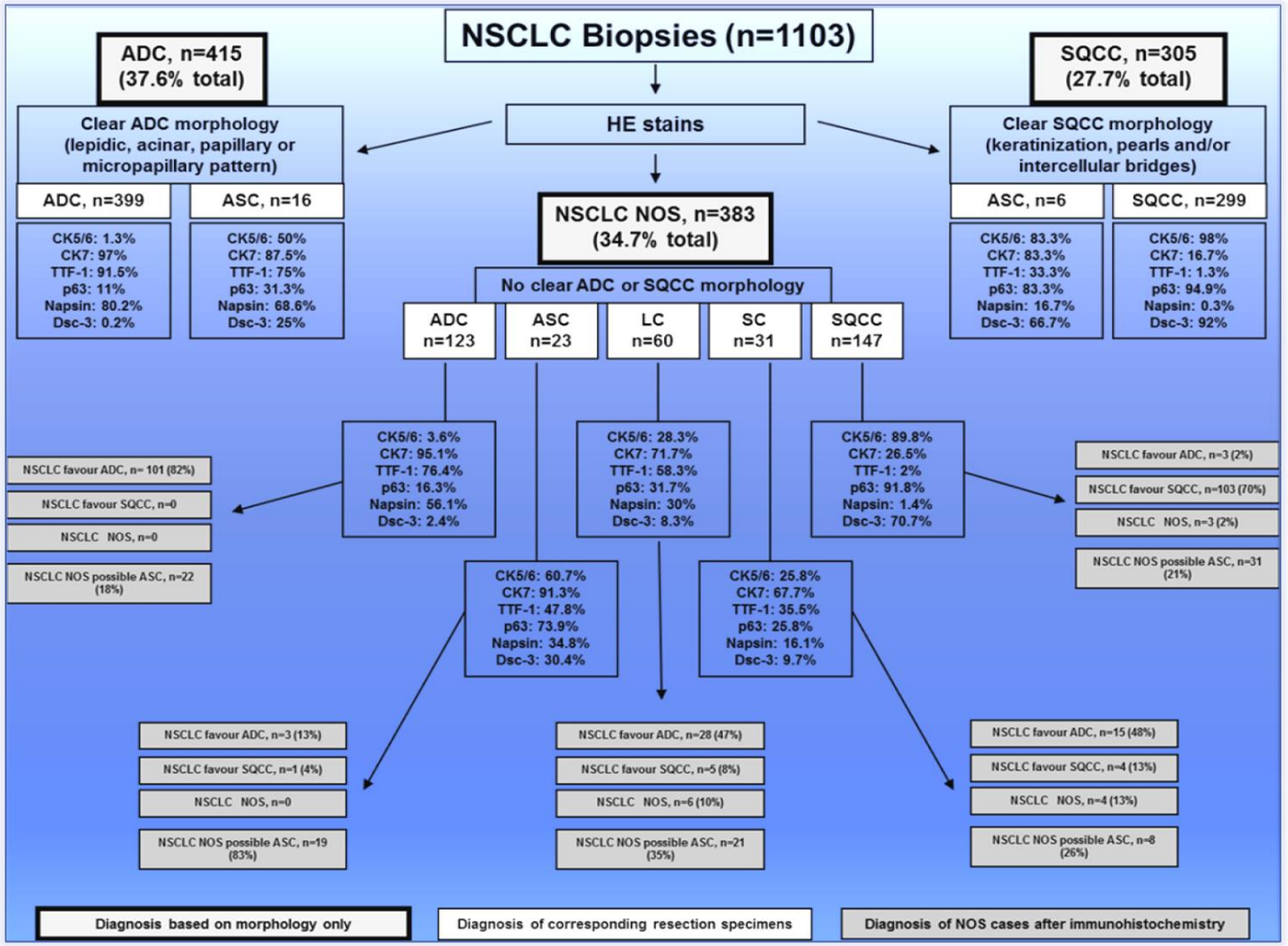
Category 2:
No more tumor tissue present, no analyses

Category3:
Critically low tumor content; valid if positive for mutation; wt result of restricted reliability



Drop-out/uncertainty: 7% of all analyses in HD (nationally best result); increases with each further test by 3-3,5 %; consequence: rebiopsy, waiting time, costs

Solution I: Rational Algorithms save Material



Warth et al., Histopathology 2012

Solution II: Improving Technology and Quality Management

- **Specific technology improvements (Extraction, Assays, IT)**
- **Improve Workflows (TAT, Reporting, Integration in Tumor Boards)**
- **Quality Management/RoundRobins/Accreditation**
- **Monitoring (positive cases, distribution, follow-up) and publication**
- **Special Case Management/Expert panels**
- **Centralisation (?)**

Heidelberg Publications

Histological Stratification: Warth et al. JCO 30 (2012) 1438-46, Eur Resp J (2012), Eur Resp J 39 (2012) 1437-42, Herpel et al., JTO 5 (2010) 2006-12; **EGFR:** Penzel et al., Virchows Arch 458 (2010) 95-8, Warth et al., Virchows Arch 460 (2012) 407-14; Gottschling et al., Lung Cancer 77 (2012) 183-91; **EML4-ALK:** Penzel et al., JTO 7 (2012)1198-9; **TS:** Herpel et al., Histopathology (2012); **Her2:** Stenzinger et al., JMD 14 (2012) 199-205; **Braf:** Andrulis et al., AJSP (2012) Apr. 22; Dietel et al., Pathologe (2012); **Kras:** Lehmann et al., Diag Mol Pathol 21 (2012) 114-9; **KIT:** Herpel et al., Anticancer Res 31 (2011)

Solution III

Innovation Next Generation Sequencing

- **Whole Genome:** complete tumor cell genome; non-focussed sequencing; low coverage
- **Whole Exome:** whole expressed transcriptome (~ 30.000 genes); low coverage
- **Panel-/targeted NGS: focussed amplification (~ 200-800 Amplikons) sequenced, high coverage; all medically relevant information**

NGS-Comparison of Methods

Parameter	Whole Genome (WGS)	Whole Exome (WES)	**Panel-/Target-Sequencing**
Little Tissue (Biopsy)	No (?ng)	No (200 ng)	Yes (< 1 ng)
Sensitivity	Low (< 80x)	Low (80x)	High (2000x)
TAT	High (>>4 wks)	High (3-4 wks)	Lower (3-5 Tage)
Paraffin/Formalin	No	No	Yes, published
Diagnostic QM	No	No	Yes (RRs, accreditation)
Diagn. Experience/-Implementation	No	No	Yes (HD, Köln)
Costs	Very high	High	Within reach
Technical Effort	Very high	Very high	Already integrated in workflow
Bioinformatics	Extremely high	Very high	In-house feasible
Diagnostic Need (Tumor)	No; science	No; science	**Necessary**

Improvement by NGS

Methodical

- **Lower drop-out rate**
  - One stop analysis: no incresed drop out by sequential analyses – less rebiopsies (costs, invasive procedure, waiting time)
  - Less grey zone results due to higher sensitivity (less uncertainty, less rebiopsy)
- **Higher sensitivity:** more resistance mutation (RAS in CRC); less unnecessary therapy (costs, unwanted effects)
- Upfront-testing **saves some tests in second and third line**
- **Potential to reduce test complexity** (amplification, translocation)

Clinical

- **Provides oncologists with all necessary information** for upfront therapy planning (clinical wish)
  - Patient information
  - Modifies therapy planning in first line
- Relevant **additional information**:
  - E.g. BRAF-mutations in CRC (not otherwise tested but invalidates EGFR inhibition)
  - Therapy planning in diagnostically unclear tumors (CUP)
  - Potential for targeted trials

Other Aspects

- Provides patients **with improved access to clinical trials**
- **Essential component of CCCs** (Umbrella-concepts)
- Basis for **registries** (monitoring; improvement of diagnostics and therapy, comparison of centers, epidemiology etc.)
- **Basis for bedside-bench research** improving diagnostic output and clinical decision making

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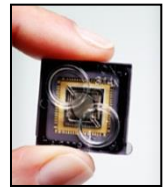
NGS-Panel Sequencing

AmpliSeq Cancer Hotspot Panel V2 (207 Amplikons)

ABL1 (4,5,6,7)	**ERBB4** (3,4,6,7,8,9,15,23)	**IDH1** (4)	**NRAS** (2,3,4)	**TP53** (2,4,5,6,7,8,10)
AKT1 (3,6)	**EZH2** (16)	**IDH2** (4)	**PDGFRA** (12,14,15,18)	**VHL** (1,2,3)
ALK **(23,25)**	**FBXW7** (5,8,9,10,11)	**JAK2** (14)	**PIK3CA** (2,5,7,8,10,14,19,21)	
APC (16)	**FGFR1** (4,7)	**JAK3** (4,13,16)	**PTEN** (1,3,5,6,7,8)	
ATM (8,9,12,17,26,34,35,36,39,50,54,55,56,59,61,63)	**FGFR2** (7,9,12)	**KDR** (6,7,11,19,21,26,27,30)	**PTPN11** (3,13)	
BRAF (11,15)	**FGFR3** (7,9,14,16,18)	**KIT** (2,9,10,11,13,14,15,17,18)	**RB1** (4,6,10,11,14,17,18,20,21,22)	
CDH1 (3,8,9)	**FLT3** (11,14,16,20)	**KRAS** (2,3,4)	**RET** (10,11,13,15,16)	
CDKN2A (2)	**GNA11** (5)	**MET** (2,11,14,16,19)	**SMAD4** (3,4,5,6,8,9,10,11,12)	
CSF1R (7,22)	**GNAQ** (5)	**MLH1** (12)	**SMARCB1** (2,4,5,9)	
CTNNB1 (3)	**GNAS** (8,9)	**MPL** (10)	**SMO** (3,5,6,9,11)	
EGFR (3,7,15,18,19,20,21)	**HNF1A** (3,4)	**NOTCH1** (26,27,34)	**SRC** (14)	
ERBB2 (19,20,21)	**HRAS** (2,3)	**NPM1** (11)	**STK11** (1,4,4/5,6,8)	



DNA Extraktion

Multiplex-PCR / Library

Proben-Multiplexing

Automatisierte emPCR

Sequenzierung auf Ion Torrent PGM/Proton

Panel Development and Roll-Out

Lung Panel

AKT1	MCL-1
ARID1A	MDM2
BRAF	MET
CBL	MYC
CCND1	NFE2L2
CCNE1	NKX-2.1
CDK6	NOTCH1
CDKNA2	NRAS
CTNNB1	PDGFRA
EGFR	PTEN
ERBB2	RB1
EYS	RBM10
FAM123B	SMAD4
FBXW7	SMARCA4
FGFR1	SOX2
FGFR2	STK11
FGFR3	TERT
HRAS	TP53
JAK2	PIK3CA
KEAP1	
KIT	
KRAS	

Breast Panel

AFF2	PIK3R1
AKT1	PTEN
APC	RUNX1
ARID1A	SF3B1
BRAF	TBL1XR1
CASP8	TP53
CBFB	MDM2
CCND1	TBX3
CDH1	TLR4
CDKNA2	GIGYF2
CTCF	RBMX
EGFR	CDKN1B
ERBB2	CDK4
FGFR1	ZNF703
GATA3	PAK1
KRAS	RPS6KA1
MAP2K4	CEP164
MAP3K1	USP36
MLL3	NR1H2
MYC	RB1
NOTCH1	PTPRD
PIK3CA	HERC1

Colon Panel

ACVR2A	SOX9
APC	SYNE1
ARID1A	TCF7L2
ATM	TGFBR2
BRAF	TP53
CASP8	
CTNNB1	
EGFR	
FAM123B	
FBXW7	
IGF2	
KRAS	
LRP2	
MLH1	
MSH3	
MSH6	
NRAS	
PIK3CA	
PTEN	
SLC9A9	
SMAD2	
SMAD4	

Molekularpathologische Begutachtung

Material
Internes Blockmaterial E-1863/14 I

Klinische Angaben
Bitte um RAS-Mutationsanalyse

Befund
Am morphologisch gesicherten und angereicherten Tumorgewebe (40 % Tumorzellgehalt) wurde eine gezielte Mutationsanalyse mittels der Next Generation Sequenzierungstechnologie (PGM; ION TORRENT) unter der Verwendung des Colon Cancer Panels V1 (180 Amplikons; u.a. N- und KRAS Exone 2, 3 und 4) durchgeführt.

Hierbei wurde die Punktmutation c.35G>A mit einer Allelfrequenz von 22 % bei einer Amplikonabdeckung (Coverage) von 3996 im Exon 2 von KRAS nachgewiesen, die zur Aminosäuresubstitution p.G12D führt.

Der Status aller anderen untersuchten Genabschnitte (s.u.) ist in unseren Datenbanken hinterlegt und kann bei Bedarf (z.B. Studienkontext) angefordert werden.

Sequenzierte Gene (Exone)

ACVR2A (9,10,11)	**ERBB2** (19,20,21)	**MSH6** (3,5)	**SMAD4** (2,3,5,6,8
APC (3,4,5,6,7,8,9,10,11,12,13,14,15,16)	**FAM123B/AMER1** (2)	**MYC** (2,3)	**SOX9** (2,3)
ARID1A (2,3,4,7,18,20)	**FBXW7** (2,3,4,5,6,7,8,9,10,11)	**NRAS** (2,3,4)	**SYNE1** (8,22,30, 82,85,12
ATM (7,8,9,11,12,20,25,29,35,38,39,40,41,42,49,50,55,58,63)	**IGF2** (2,3)	**PIK3CA** (2,3,5,8,9,10,14,19,21)	**TCF7L2** (5,9,10,14)
BRAF (11,15,16)	**KRAS** (2,3,4)	**POLE** (9,13,14,32,33)	**TGFRB2** (5,6,7)
CASP8 (3,9,10)	**LRP2** (49,53)	**PTEN** (1,2,5,6,7,8,9)	**TP53** (4,5,6,7,8,9,10)
CTNNB1 (3,5,6)	**MLH1** (1,2,6,7,8,9,14,16)	**SLC9A9** (3)	
EGFR (18,19,20,21)	**MSH3** (7)	**SMAD2** (8,11)	

since 02/14 Panel sequencing in regular diagnostics for CRC, GIST, breast and lung cancer, and CUP

NGS-Panelsequencing in Routine-Diagnostics (Heidelberg)

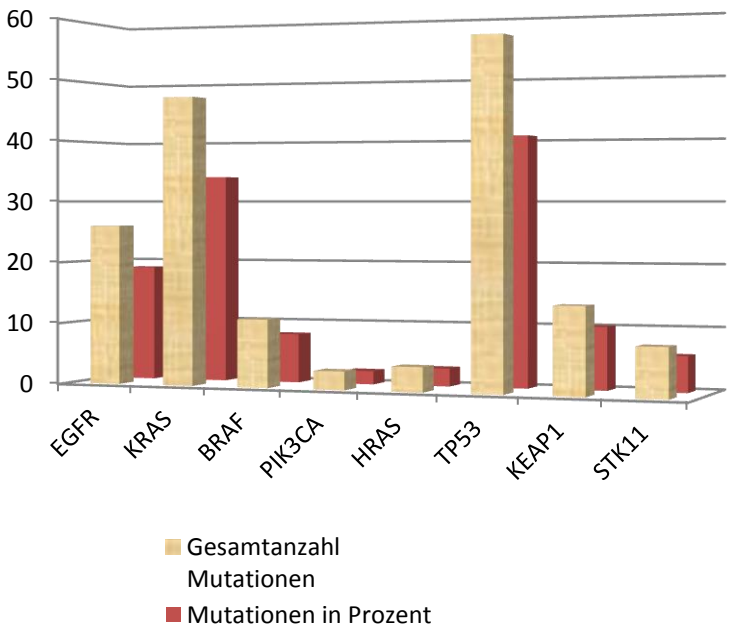
NGS-Sample-Statistics (NCT)

01.03. - 30.05.2014

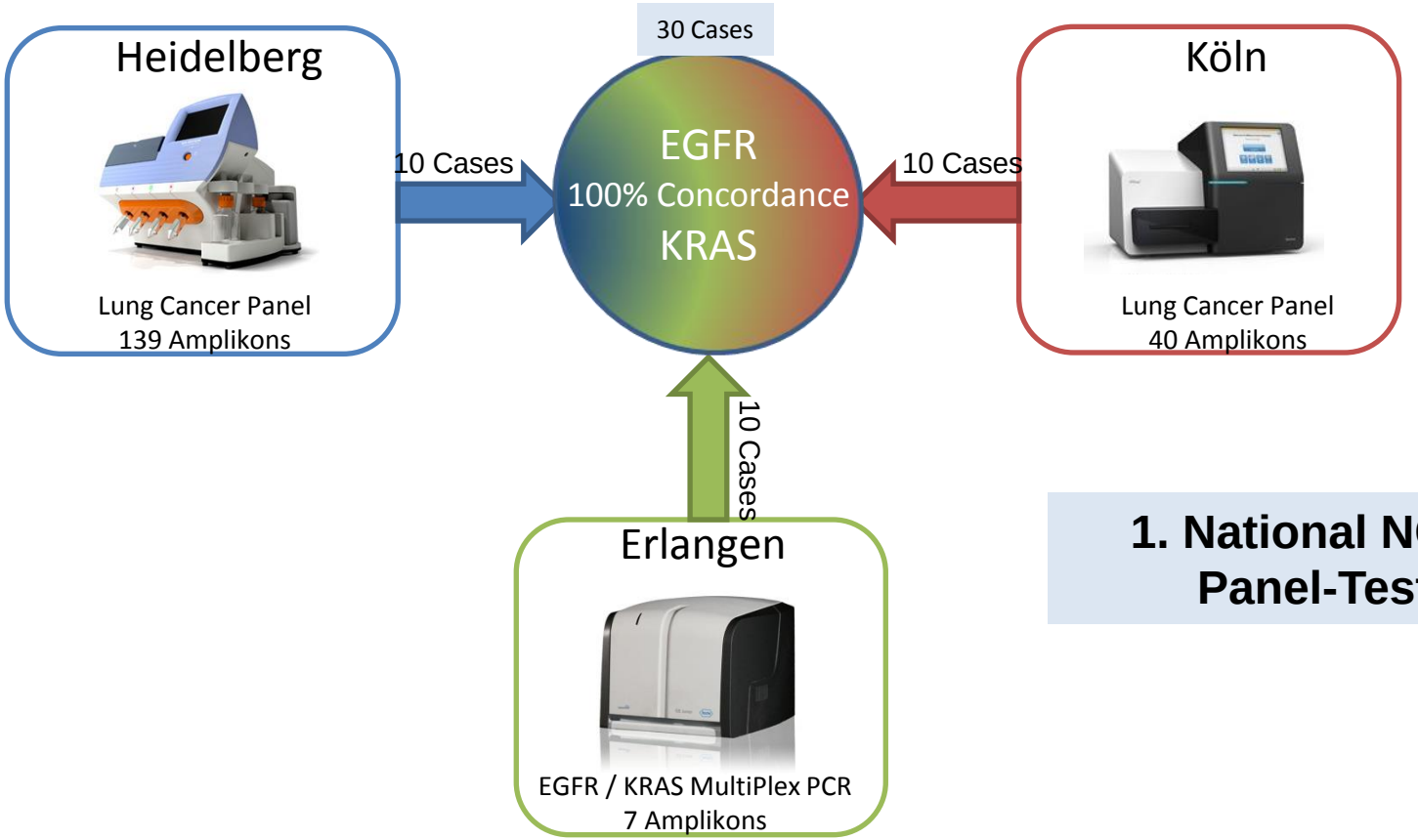
- Lung: 138 Cases
- Colon: 76 Cases
- Melanoma: 38 Cases
- GIST: 12 Cases
- Others (CUP): 65 Cases

Total: 329/3 months

Example Lung



Pre-RR: QA Panel Sequencing



1. National NGS-Panel-Test

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DKTK-NGS-FFPE Trial (7 Sites)

Comparison of NGS technologies for FFPE materials

- Comparability of different sequencing sites
- Comparability of different NGS platforms
- Comparability of different DNA extraction (FFPE) protocols
- Comparability of different gene panels (multiplex PCRs)
- Comparability of different bioinformatics procedures

LOCAL MICRODISSECTION & DNA EXTRACTION

Local micro-dissection

Local DNA-extraction

Ion AmpliSeq™ Cancer Hotspot Panel v2

3x Ion Torrent PGM

KDR	PTPN11
KIT	RB1
KRAS	RET
MET	SMAD4
LH1	SMARCB1
PL	SMO
OTCH1	SRC
PM1	STK11
RAS	TP53
DGFRA	VHL
K3CA	
TEN	

TrueSeq Amplicon Cancer panel

ABL1	EGFR	GNAS	MLH1	RET
AKT1	ERBB2	H		
ALK	ERBB4	H		
APC	FBXW7	ID		
ATM	FGFR1			
BRAF	FGFR2			
CDH1	FGFR3			
CDKN2A	FLT3			
CSF1R	GNA11			
CTNNB1	GNAQ			

2 x Illumina MiSeq

DNA provided to the NGS sites

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Detection of the predefined mutations by amplicon NGS

Tumor	Gene	PGM	PGM	MiSeq	MiSeq	PGM
ColonCa 1	KRAS	✔	✔	✔	✔	✔
ColonCa 2	KRAS	✔	✔	✔	✔	✔
ColonCa 3	KRAS	✔	✔	✔	✔	✔
ColonCa 4	KRAS	✔	✔	✔	✔	✔
ColonCa 5	KRAS	✔	✔	✔	✔	✔
BreastCa 1	PIK3CA	✔	✔	✔	✔	✔
BreastCa 1	PIK3CA	✔	✔	✔	✔	✔
BreastCa 2	PTEN	✔	✔	✔	✔	✔
BreastCa 3	PIK3CA	✔	✔	✔	✔	✔
BreastCa 3	PIK3CA	✔	✔	✔	✔	✔
BreastCa 4	PIK3CA	✔	✔	✔	✔	✔
BreastCa 5	PIK3CA	✔	✔	✔	✔	✔
LungCa 1	EGFR	✔	✔	✔	✔	✔
LungCa 2	EGFR	✔	✔	✔	✔	✔
LungCa 3	EGFR	✔	✔	✔	✔	✔
LungCa 4	EGFR	✔	✔	✔	✔	✔
LungCa 5	EGFR	✔	✔	✔	✔	✔

Results with the DNA provided to the NGS sites

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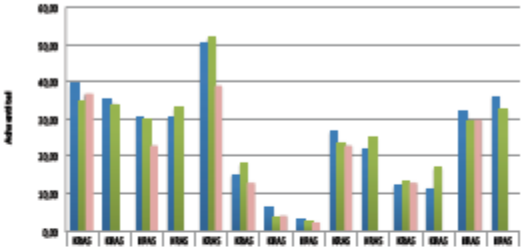
Conclusions DKTK NGS Trial

- Multiplex-PCR amplicon-based NGS is an excellent tool for detection of mutations in FFPE specimens
- High coverage (average 2000x)
- High sensitivity (1% tumor cells)



- Great homogeneity within the NGS platforms specially PGM)
- Careful consideration of gene panels and DNA extraction methods
- Consideration of NGS platform specific characteristics

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Obstacles to Implementation of Panel-NGS in Clinical Diagnostics

- Does not cover all predictive tests (60-80%)
- Does not cover all positive cases; DNA is surrogate marker
- Requires justification by sufficient molecular targets per case
- Companion diagnostics principle (US)
- Rejection of NGS by authorities (e.g. Germany)
- No/insufficient refunding
- Principle of indication bound diagnostics
- Limited availability for diagnostic use (few sites)
  - High investment, rapid technology changes
  - Personel (TA, bioinformatics, diagnostic PhDs)

‚Liquid Biopsy‘

Definition: Molecular analysis of informative molecules (mainly nucleic acids) from body fluids (mainly blood)

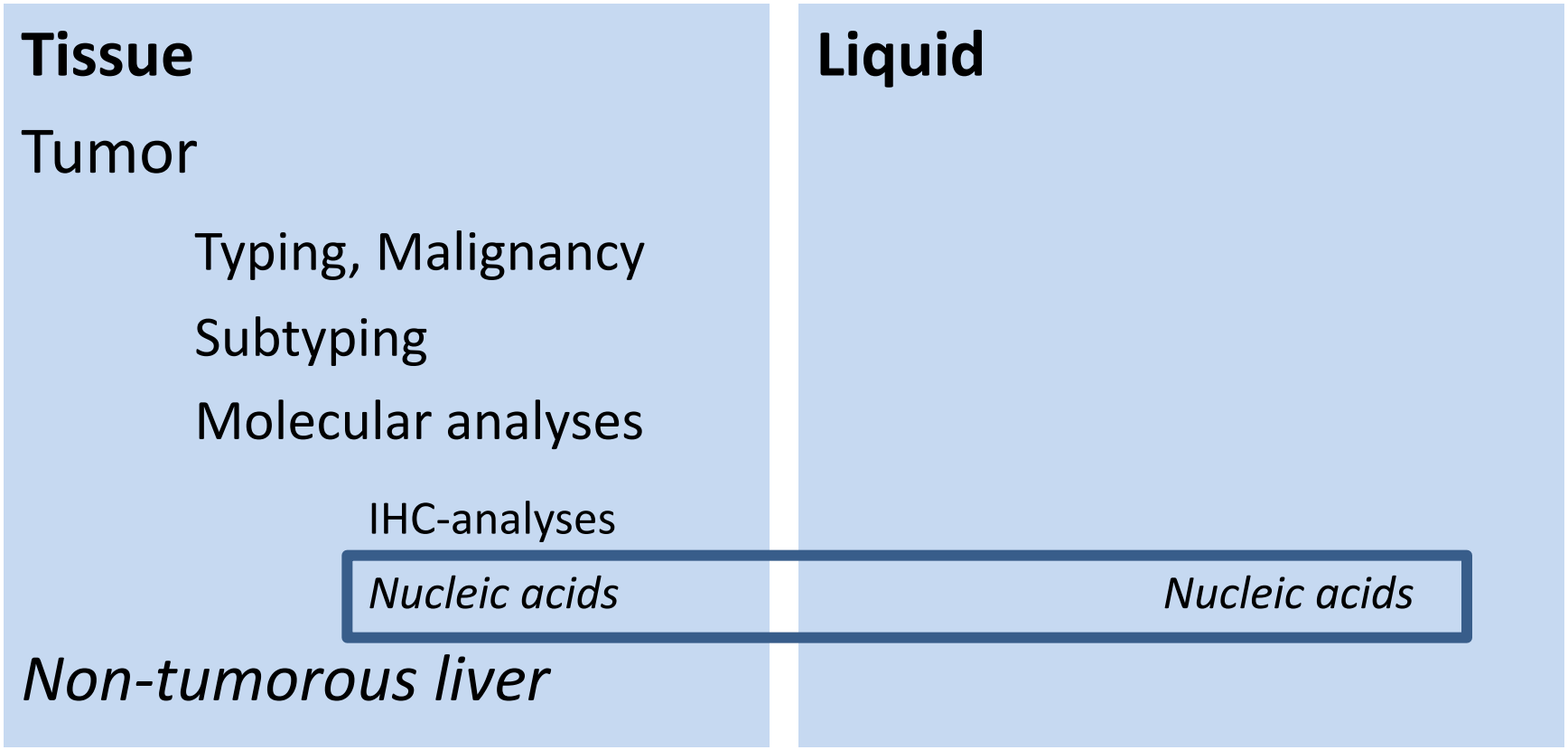
Aims: Early detection, diagnosis, predictive testing and follow-up (esp. Cancer)

Sources:

- **Circulating Tumor Cells (CTCs)**
- **Cell-free DNA (cfDNA)/circulating tumor DNA (ctDNA)**
- *Exosomes*

Expert Statement of German Society of Pathology (DGP)

Tissue vs. ‚Liquid Biopsy' in Tumor Diagnostics



‚Liquid Biopsy' is a *misnomen*, suggesting equal level and quality of procedure and information obtained

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Circulating Tumor Cells (CTCs) What do they represent?

- Which tumor cells enriched and which not (are they tumor cells? EpCAM selection)? - variable
- Which part of the tumor is represented?
    - Primary vs. metastasis - unknown
    - Relation of CTCs to CSC? - undefined
    - Site specificity - unknown
- Presence in non-oncological patients - unclear significance
- Quantitative representation of tumor relevant changes (mutations, resistance phenotype) – not present
- Other unclear situations
    - Double tumors – not accessible

We have currently **no information which tumor cell populations and which tumor characteristics we measure with CTCs** with which reliability. This is likely to **remain highly variable and non-standardised for diagnostic purposes**

Circulating Tumor Cells (CTCs)

- **Low concentration** in peripheral blood (0-few 100 cells/10 ml blood); only 1,4% of stage IV breast cancer patients >500 CTCs /7,5 ml blood! (Bacelli et al, Nat Biotechnol, 2013)
- Presence **stage specific** (Bettegowda et al., Sci Transl Med, 2014)
    - Stage I: 47% over all entities
    - Stage IV: 82% over all entities
- Presence **entity specific** (stage IV) (Bettegowda et al., Sci Transl Med, 2014)
    - CRC: ~100%
    - Prostate: ~40%
    - Kidney: ~40%
    - Brain: <10%
- Presence **location specific** (CRC: CTCs in mesenterial veins > central veins) (Rahbari et al., Ann Surg Oncol, 2012)
- Presence in **non-oncological patients** (CED, fibrous mastopathy)?? (Pantel et al., Clin Chem, 2012)

CTCs are highly variable and **not useful for early detection of cancer.** Due to significantly lower sensitivity and lack of sufficient and standardized acquisition they are **not a useful source for any kind of tumor diagnostic procedure** (typing or prediction)

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Blood-derived Nucleic Acids

- **Concentration:** 0-100 ng/ml blood
- **Purification:** Affinity chromatography
- **Source:** neoplastic and non-neoplastic cells
- **Condition:** necrosis? apoptosis? vital cells?
- **Half-life:** ~1.5 h

Amplification of Blood-derived DNA

Digitalized Signals

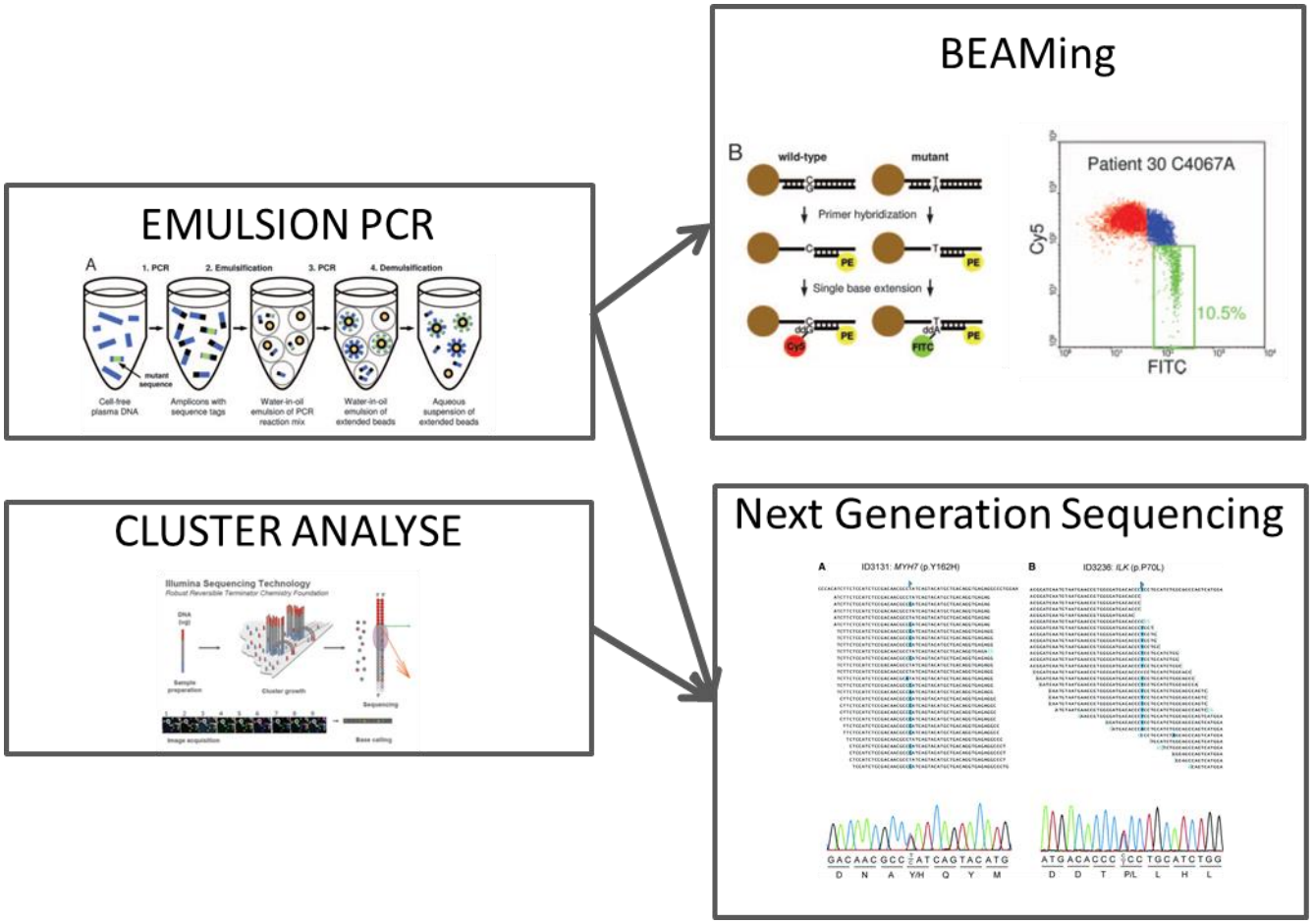
BEAMing PCR (Beads-Emulsion PCR-Amplification-Magnetic Beads) (Diehl et al., 2005, 2006, Li et al., 2009)

NGS (Li et al., 2009; Lianidou and Markou, 2011, Heitzer, 2013) (Ion Torrent, Illumina, 454)

- **Independent single template amplification of signals**
  - Emulsion PCR (BEAMing, Ion Torrent, 454)
  - Cluster (Illumina)
- **Detection**
  - FACS (BEAMing)
  - Sequencing (Ion Torrent, Illumina, 454)

Highly amplyfying methodologies: prone to contamination

Detection Strategy for Blood-derived DNA



Combination of 2 highly amplifying technologies

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Blood-derived Nucleic Acids Sensitivity in Tumor Diseases

Stage-dependent:

- Early Stage: low (47%)
- Stage IV: moderate (82% over all entities vs. Tumor Biopsy: 96.5%)

Entity-dependent (Stage IV):

- CRC: ~100%
- Ovarian-Ca: ~100%
- Prostate-Ca: ~40%
- Kidney-Ca: ~40%
- Brain-Tumors: <10%

Bettegowda et al., Sci Transl Med 2014

Not useful for screening purposes

Too low for regular clinical diagnostics; needs evaluation for every specific condition

Consistency between PT and Blood DNA

- CRC/KRAS-codon12/13 mutations: sensitivity 87.2%; specificity: 99.2% (Bettegovda et al., 2014)
- Exom-sequencing of breast, lung, ovarian cancer ctDNA compared to tissue: 60% of mutations detected in breast cancer; 19% in ovarian cancer (Murtaza et al., 2013)
- Correlation of BRAF mutation in melanoma tissue and cfDNA: 84% (V600E) – 97% (V600K) (Ascierto et al., 2013)

Variable, due to complexity of mutation and entity?

Would require extensive entity and assay specific validation

Excluded/critical Clinical Conditions

- Double malignancy around the same date (5-10% of patients); may be unknown!
- Co-occurence of premalignant neoplasia
  - CTC and ctDNA (?) found in nonmalignant conditions
  - Extremely relevant and frequent in HCC (HBV! and other high risk conditions)
- Acute therapeutic intervention (TACE, Rx etc.) (non-repesentative?); significant inflammation?

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Refametinib in RAS-mutated HCC (Phase II; KRAS-BEAMing-Detection)

- The sensitivity in HCC is probably moderate; the specificity can not be determined (e.g. premalignant lesions, second malignancy)
- Lower sensitivity (~400% higher diagnostic drop-out compared to tumor biopsy); drop-outs are not recognised!
- Lower sensitivity harms recruitment but principally not trial success (approval can be reached) and is balanced by easier recruitment
- Lower sensitivity is deleterious for clinical success/patient recruitment once approval may be granted
- Insufficient, not broadly implemented test will lead to diagnostic and subsequent recruitment failure

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Blood-derived Nucleic Acids Analyses Diagnostic Applications

Potential

- Appearance of resistance mutations ? (when to react?)
- Correlation to tumor load? – monitoring of response/early response prediction?
- Repetitive analyses possible!

Not validated!

Limitations

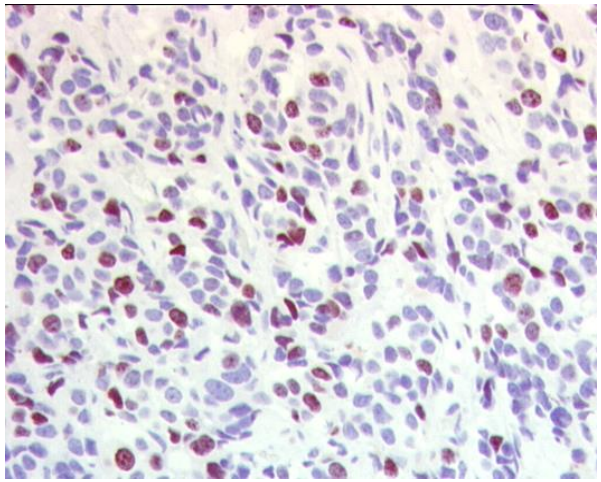
- Insufficient sensitivity - not applicable for primary diagnosis/ molecular analysis
- Relevant (unknown) clinical conditions excluded
- Heterogenous, non-comparable, not validated and quality assured technologies
- All current validation based on baseline tumor biopsy
- only amenable to NA-based analyses

Conclusions

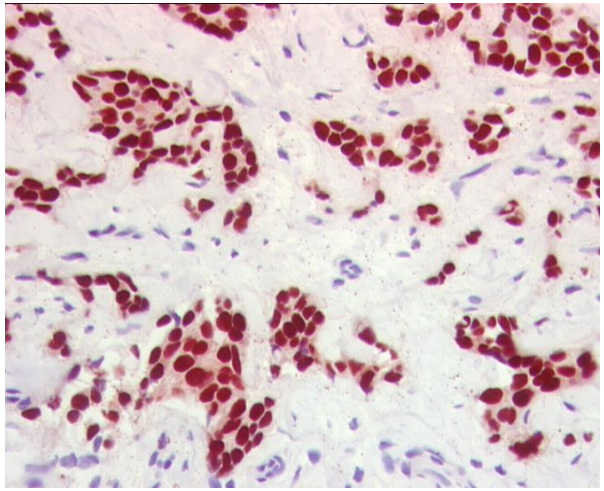
- ‚Liquid Biopsy' is unable to replace diagnostic tumor biopsy
- ‚Liquid Biopsy' is not ready for any diagnostic application
- ‚Liquid Biopsy' provides significant research application (CTC) and on the long run after significant improvement and validation may have limited diagnostic application (response/resistance; drug selection)
- Many tumors are poorly suited for diagnostic ‚Liquid Biopsy' (high and uncontrollable load of premalignant lesions)

Prognosis: Commercial interest threatens to beat scientific and clinical evidence

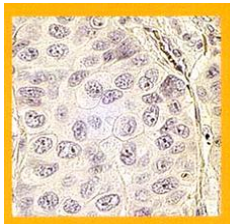
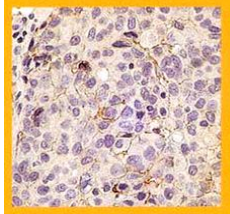
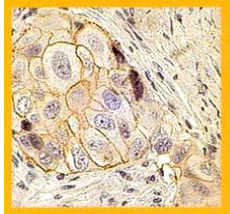
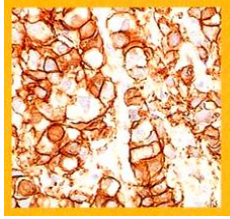
Immuno-Tests



Ki67: yes/no

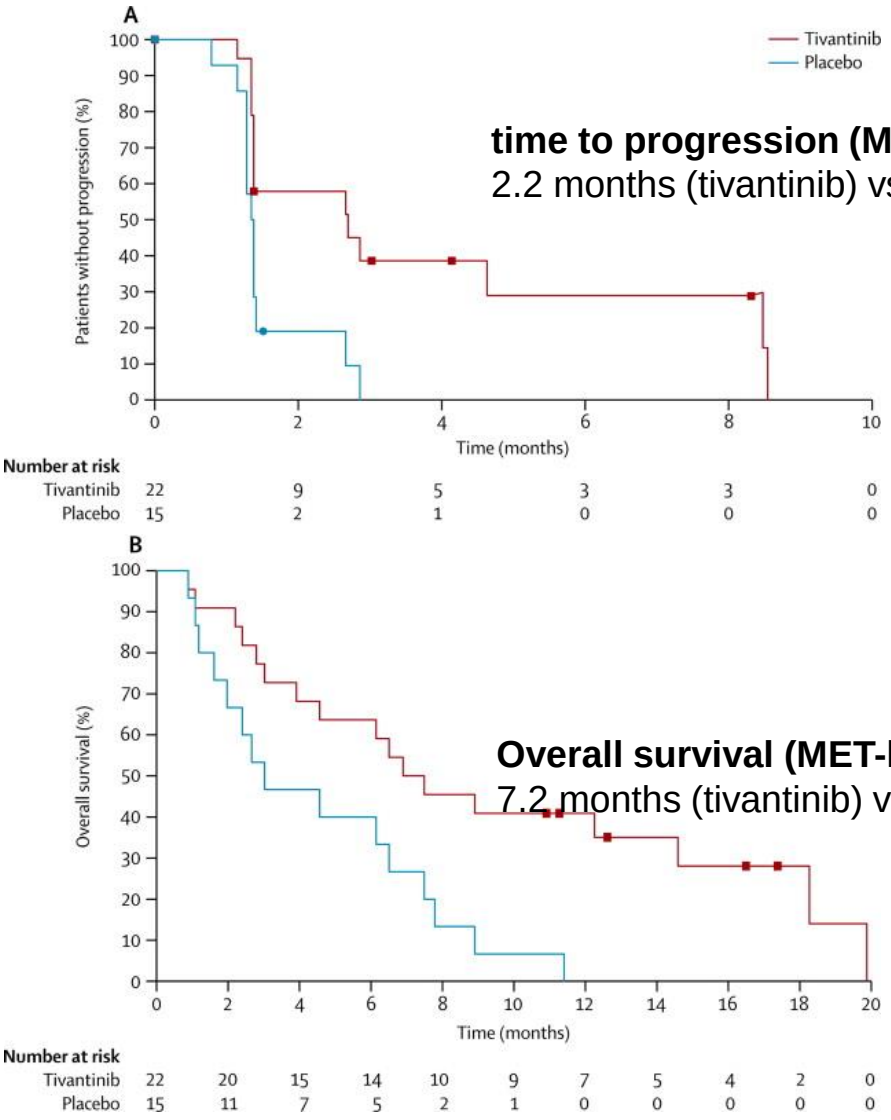


ER/PR: yes/no; intensity

	Keine Färbung zu sehen oder **weniger als 10%** der Tumorzellen zeigen eine membranständige Anfärbung.	0	Negativ
	Eine schwache oder kaum sichtbare Membranfärbung ist in **mehr als 10%** der Tumorzellen zu sehen. Die Zellen zeigen eine nur **unvollständige** Membranfärbung.	1+	Negativ
	Eine schwache bis moderate **komplette** Membranfärbung wird in **mehr als 10%** aller Tumorzellen festgestellt.	2+	Schwach Positiv
	Eine **starke**, die komplette Membran umfassende Färbung wird in **mehr als 10%** aller Tumorzellen beobachtet.	3+	Stark Positiv

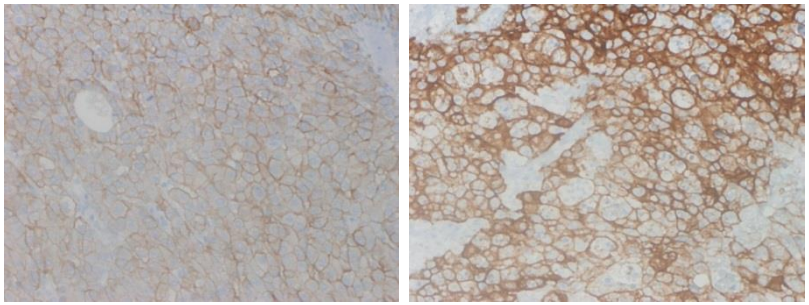
Her-2:intensity and continuity of membranous signal, # of positive cells

Tivantinib: Expression makes the Difference



time to progression (MET-high):
2.2 months (tivantinib) vs. 1.4 months (placebo)

Immunohistochemistry (MET-high):
at least 2+ in at least 50% of tumor cells



Overall survival (MET-high):
7.2 months (tivantinib) vs. 3.8 months (placebo)

Santoro et al., Lancet Oncol 2013

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Slide Information Storage

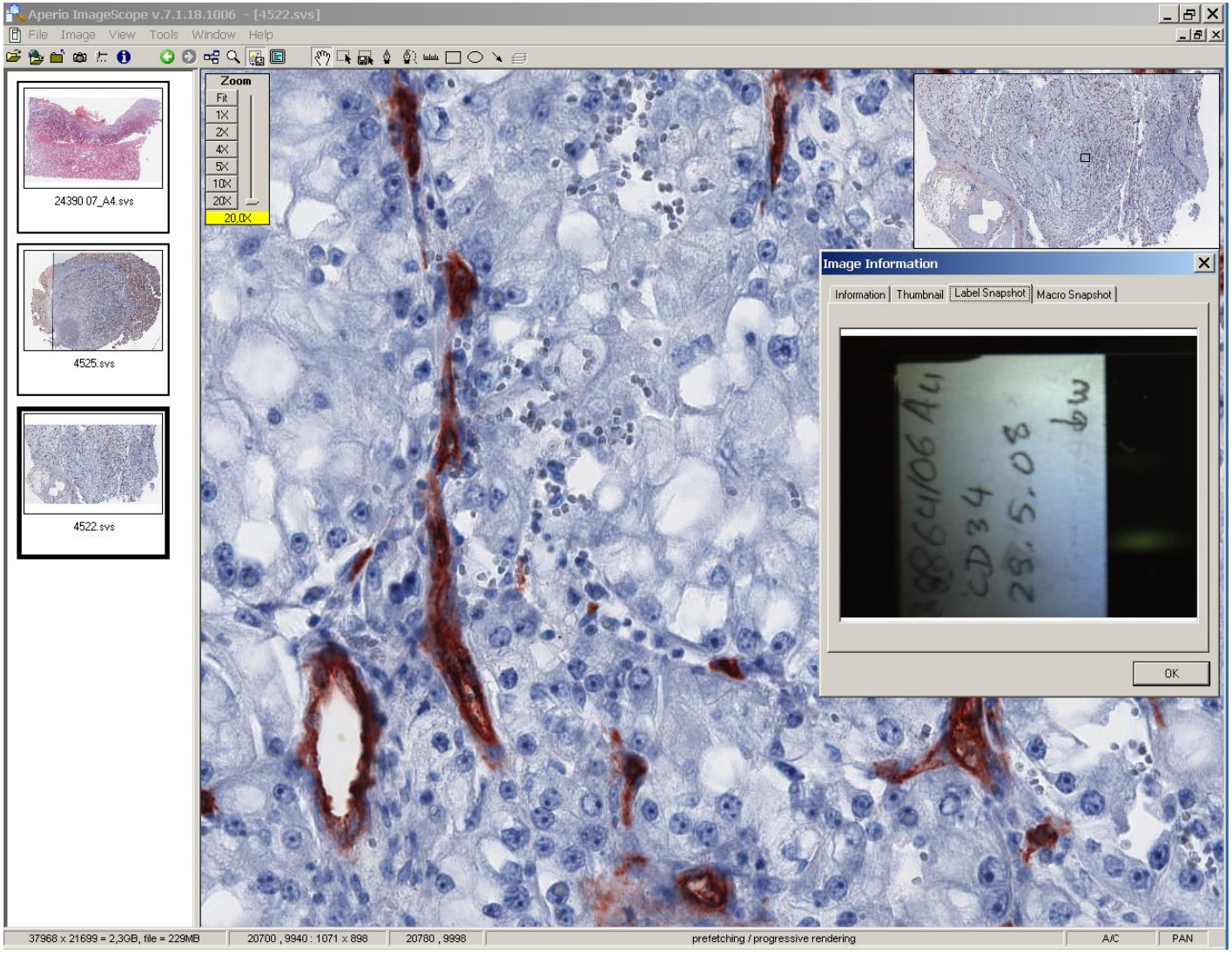

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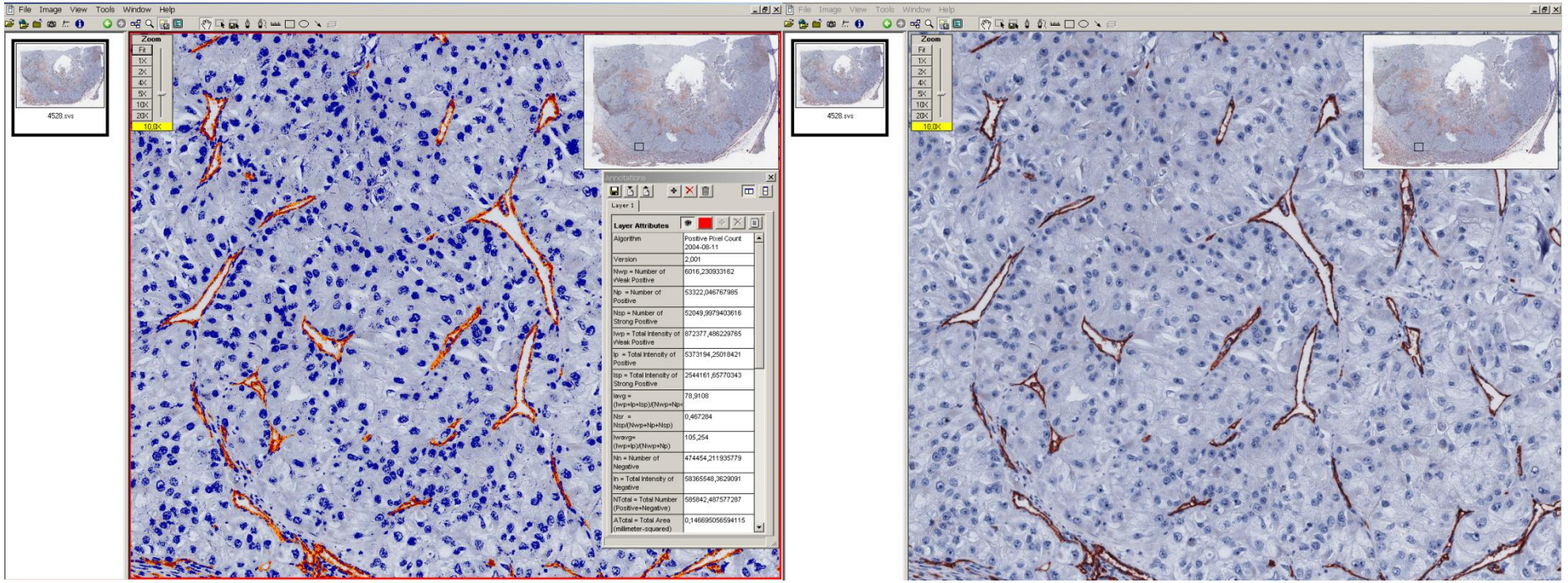
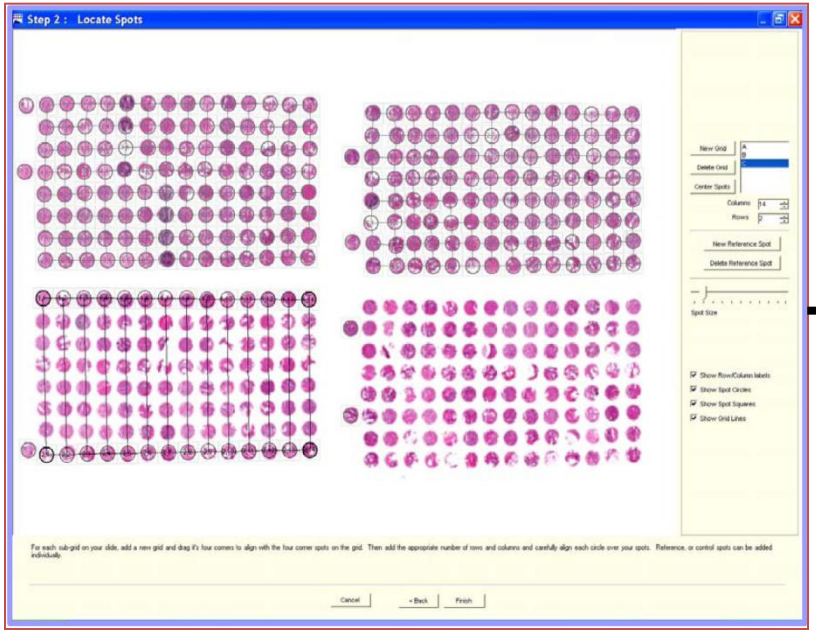
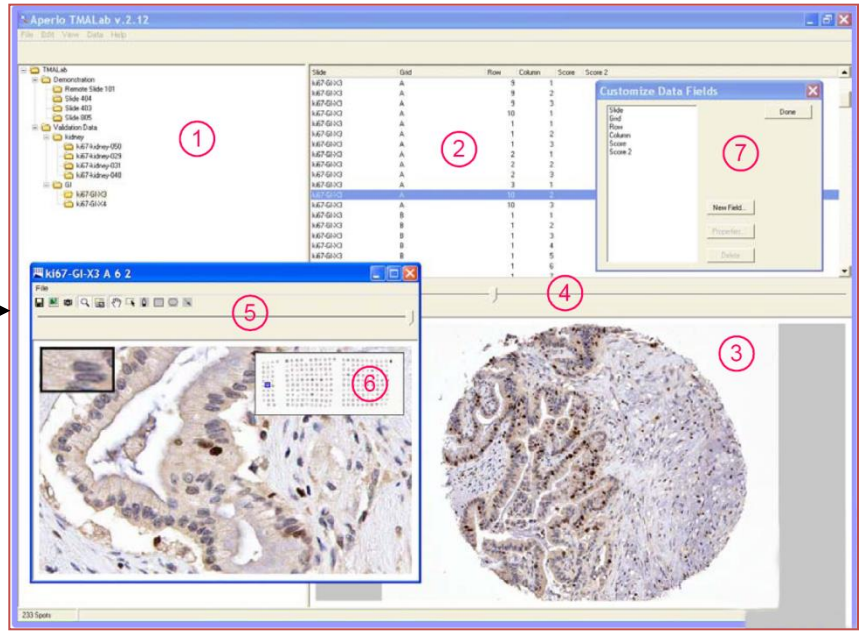
Image Analysis



Macher-Goeppinger et al., Neoplasia 10 (2008) 1049-56

Digital Data Acquisition and Analysis





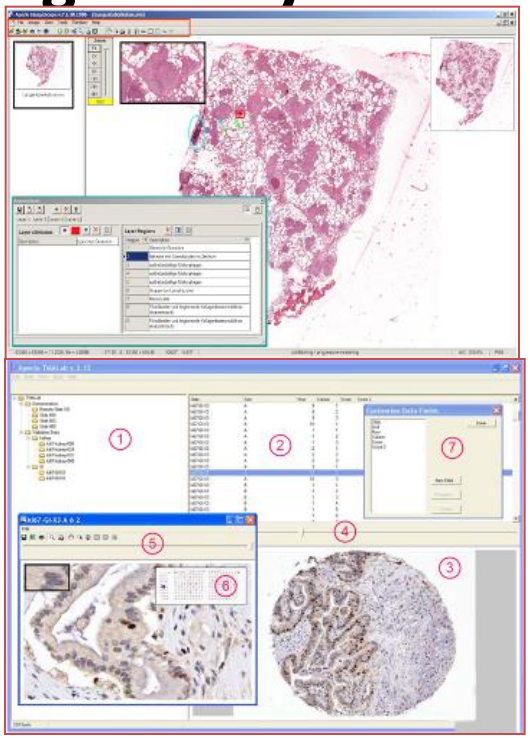
TIGA Center – VM and Image Analysis

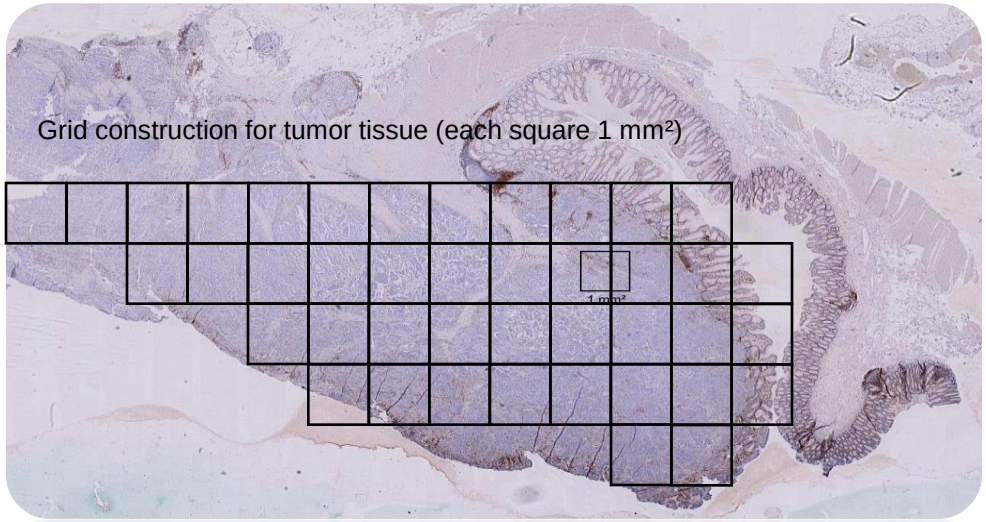
Goals:

Standardized „read-out" of FISH/IHC in clinical studies

- Identifying positive patient subgroups
- Significant biomarkers?
- Borderline cases
- Reclassification/revisiting guidelines

=> Objective and automated quantification of histological classifiers (trials and routine diagnostics) !





Halama et al.,Tumor Maps: Quantification of Prognostic Immune Cell Markers in Colorectal Cancer Using Whole Slide Imaging, *Anal Quant Cytol Histol*, 2010

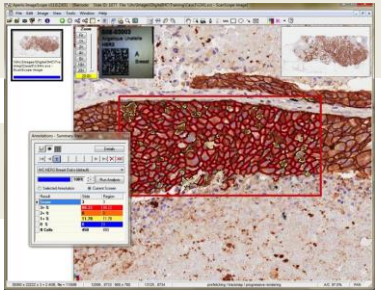
Diagnostic Applications

Indications

- Proliferation index (endocrine/mammary)
- Receptor expression (ER, PR, Her2)
- Novel markers
- Trial associated analyses!
- Cytology
- Histology parameters

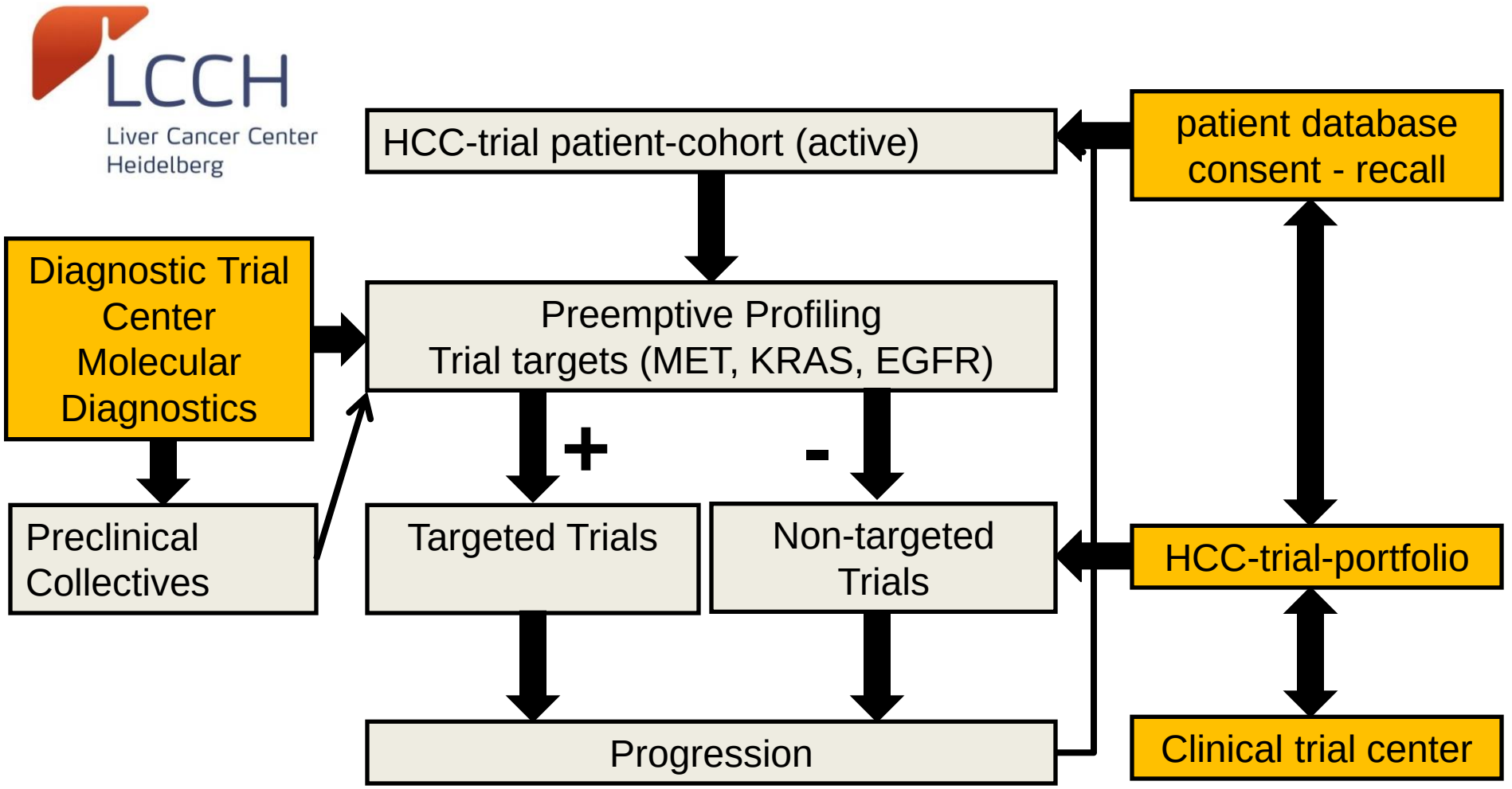
Challenges

- Tumor entity adjusted tumor-stroma segmentation
- Technology (IHC, FISH, CISH)
- Signal type (yes/no, intensity, subcellular compartment, distance etc.)
- Area selection
- Standard
- Artifact recognition



Nevertheless, this is the proof of principle!

Umbrella Concept



17 active HCC Trials

Advantages Umbrella Concept

- Rapid recruiting for clinical trials
- Optimized patient allocation in trials
- Improved calculation (industry, planning)
- Improved patient management
- Well-suited for networks (win-win)
- Scalable

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Conclusions

- NGS (Panel sequencing) offers significant diagnostic, clinical and technological improvement over single tests and is ready for application
- Liquid biopsy has potential for research but is ready for diagnostic use and is in principle inferior to tissue based analyses; there are many unsolved technological and diagnostic issues
- Vitual microscopy combined with digitalized image analyses has great potential to improve IHC- and FISH based analyses to generate quantitative data
- Umbrella concepts combine comprehensive molecular analyses for clinical and trial purposes with patient management strategies. They are mandatory for strong oncology centers for improving patient and trial management

Thank You!

IPH

- Molecular Diagnostic Center (W. Weichert, R. Penzel & Coworkers)
- Diagnostic Trial Center (W. Weichert, T. Ruf & Coworkers)

National Center of Tumor Diseases (NCT)

Clinical Partners

Tissue Imaging and Analysis Center Heidelberg (TIGA, N. Grabe)

German Consortium for Translational Cancer Research (DKTK)

German Society of Pathology (DGP) Working Group ‚Liquid Biopsy' (E. Dahl, S. Lassmann)

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Circulating Tumor Cells (CTCs)
Translational Applications

Xenopatients

- In vivo amplification for research purposes
  - Mechanistic analyses
  - Interfering mechanisms
  - (functional) imaging
- In vivo amplification for clinical purposes
  - Drug testing?? (representativity)
  - Biomarker analysis/development

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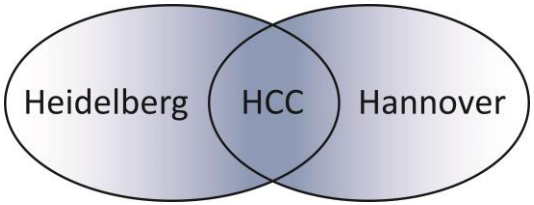
Quality Assessment in Panel Sequencing?

- Accreditation Institute (DAkkS)
- Round Robin Trials (QUiP)
- Inter-Center-Optimisation (DKTK)
- Preclinical Validation (DKTK)

Thank you!

- SFB/TRR77 Liver Cancer (Heidelberg/Hannover)
- Liver Cancer Center Heidelberg (LCCH)
- Institute of Pathology, University Hospital Heidelberg (IPH)
  - Molecular Hepatopathology Research Team
  - Diagnostic Trial Center Heidelberg
  - Molecular Diagnostic Center
- Tissue Imaging and Analysis Center Heidelberg (TIGA)
- Virtual Liver Consortium



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Liver Cancer Center Heidelberg

Adaptation Single Marker: RAS in CRC

Cetuximab/Panitumumab

KRAS - Codon12/13

KRAS - Codon 61

NRAS - Codon 12/13/61

	Pmab + FOLFOX (n=320)	FOLFOX (n=321)	HR (95% KI)	p-Wert
WT *RAS*[a], n	259	253	-	-
Medianes OS – Monate (95% KI)	**26,0** (21,7–30,4)	**20,2** (17,7–23,1)	0,78 (0,62–0,99)	0,04
Medianes PFS - Monate (95% KI)	10,1 (9,3–12,0)	7,9 (7,2–9,3)	0,72 (0,58–0,90)	<0,01
MT *RAS*[b], n	272	276	-	-
Medianes OS – Monate (95% KI)	**15,6** (13,4–17,9)	**19,2** (16,7–21,8)	1,25 (1,02–1,55)	0,04
Medianes PFS - Monate (95% KI)	7,3 (6,3–7,9)	8,7 (7,6–9,4)	1,31 (1,07–1,60)	0,01

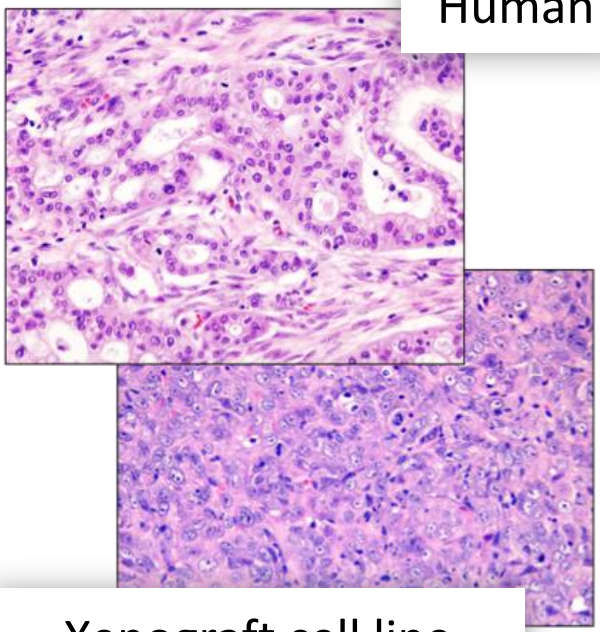
a: Wildtyp in *NRAS* und *KRAS* Exons 2, 3, 4; b: Mutation in den *KRAS* oder *NRAS* Exons 2-4 (*RAS* Bestimmungsrate 90%)

4 Amplikons

12 Amplikons

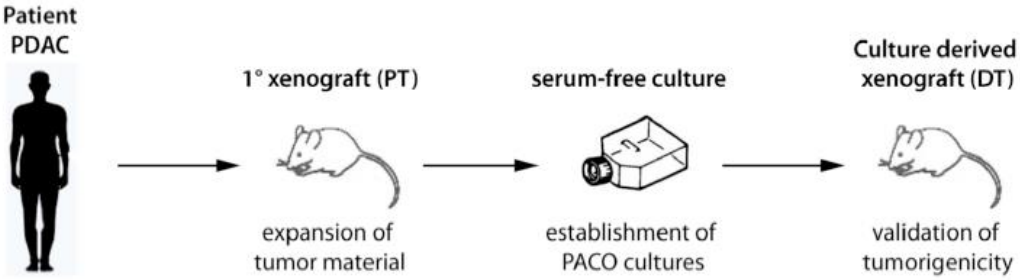
Oliner K, et al. ASCO 2013 (poster discussion) Abstract #3511.

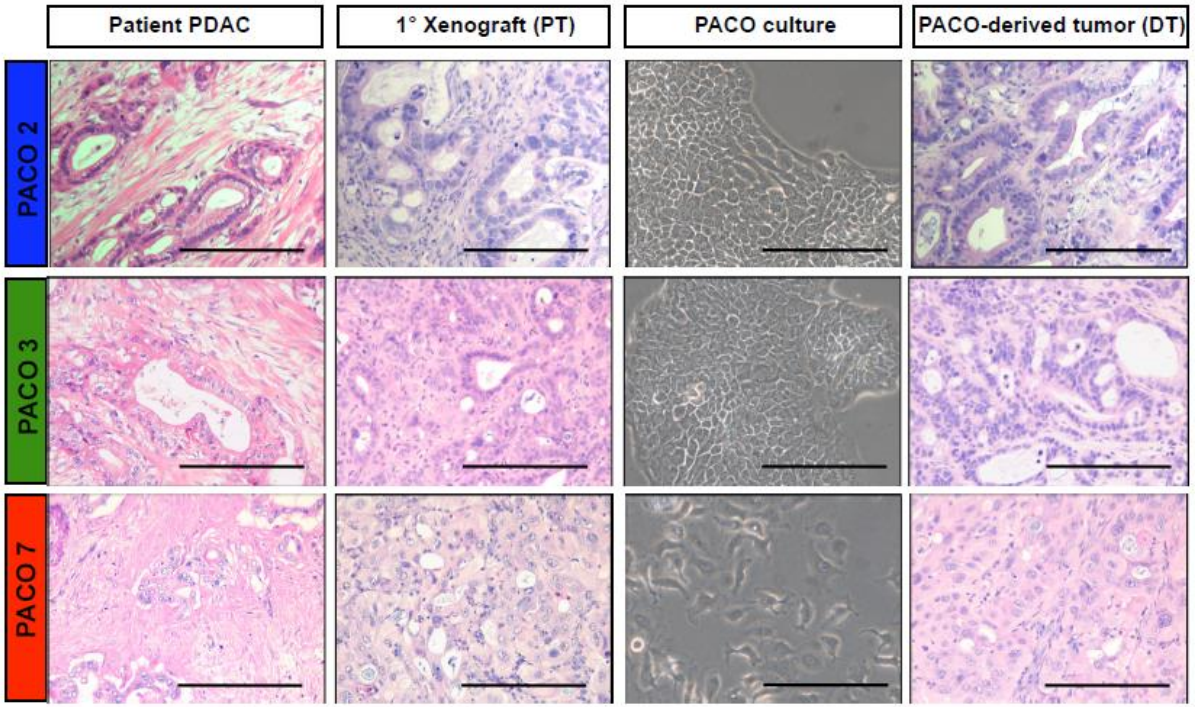
CTC-derived Tumors



Human

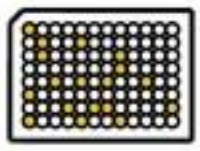
Xenograft cell line



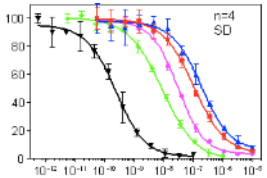


Different Sensitivity for Chemotherapy

Serial dilutions in quadruplicates

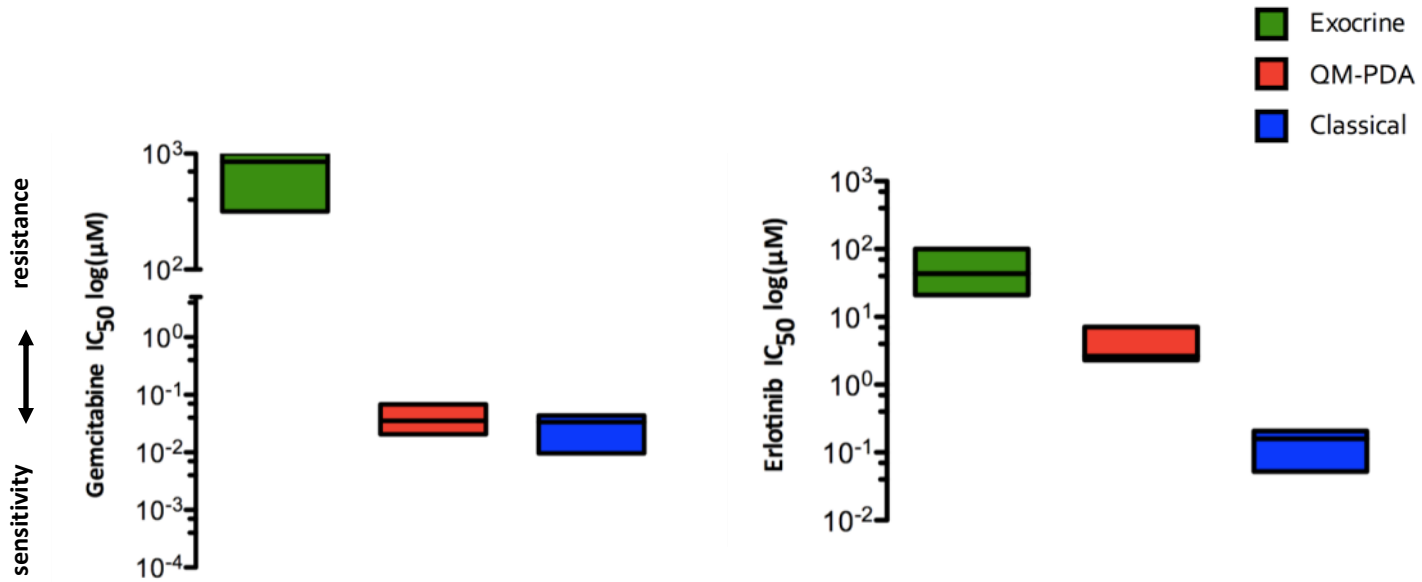


Calculation of individual IC50 values

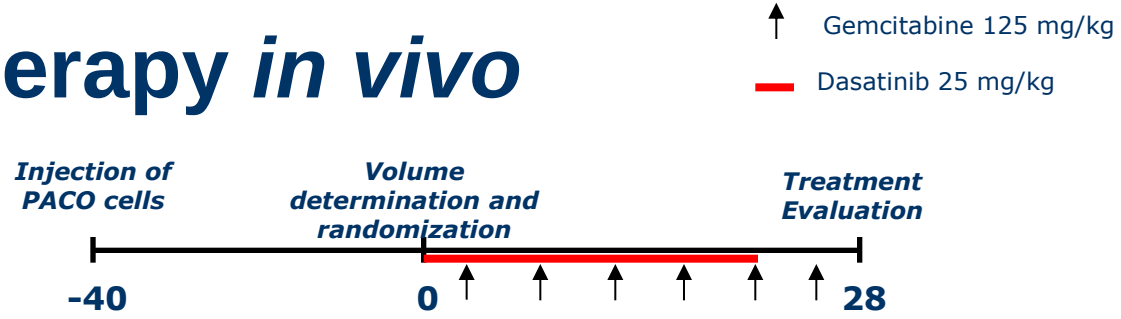


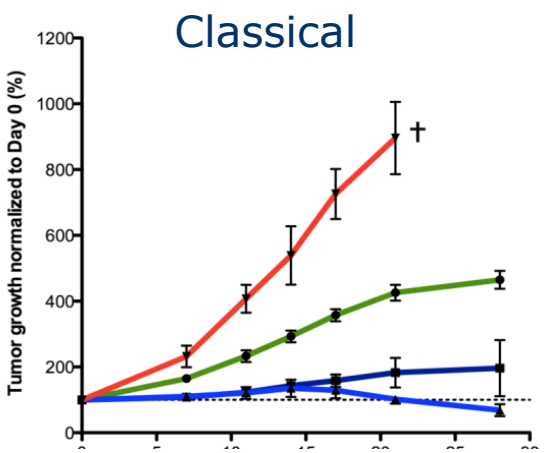
Chemotherapeutic

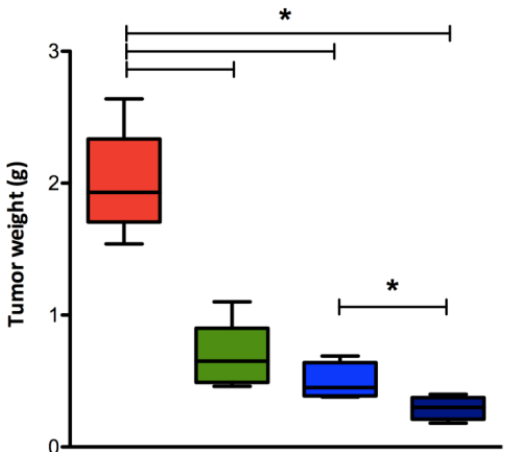
Cell Viability Assay

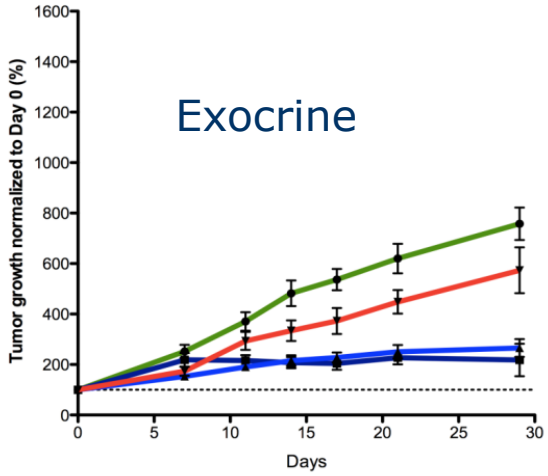


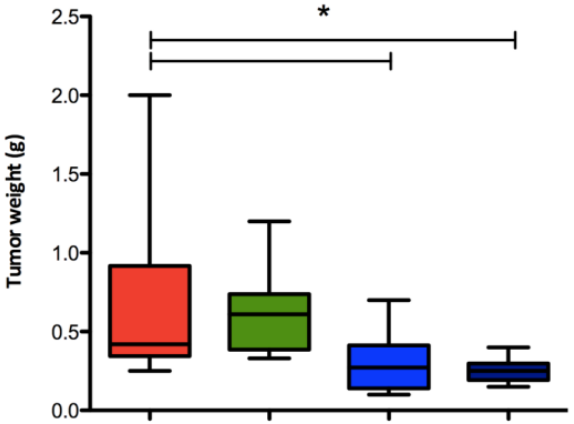
Chemotherapy *in vivo*



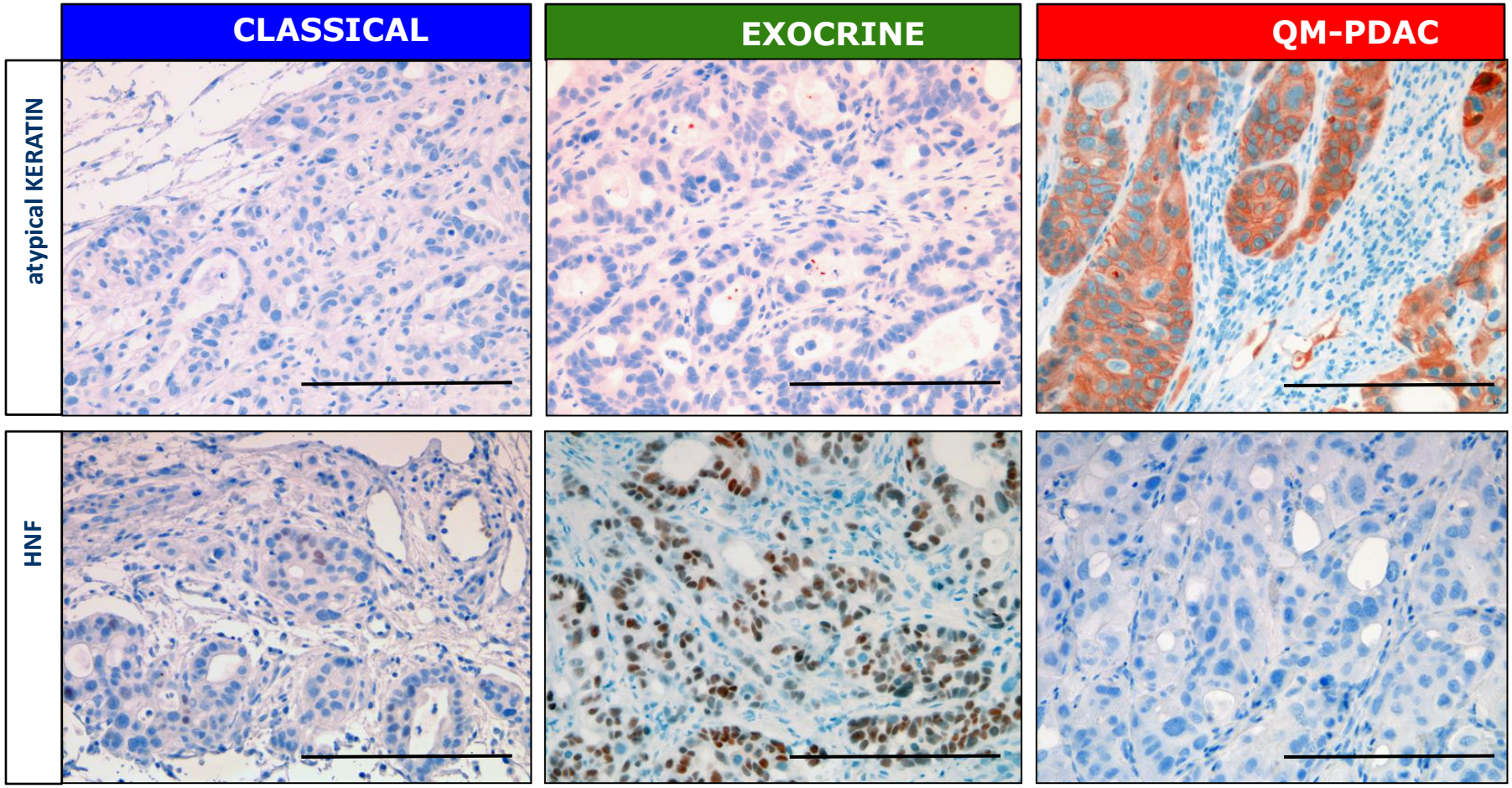




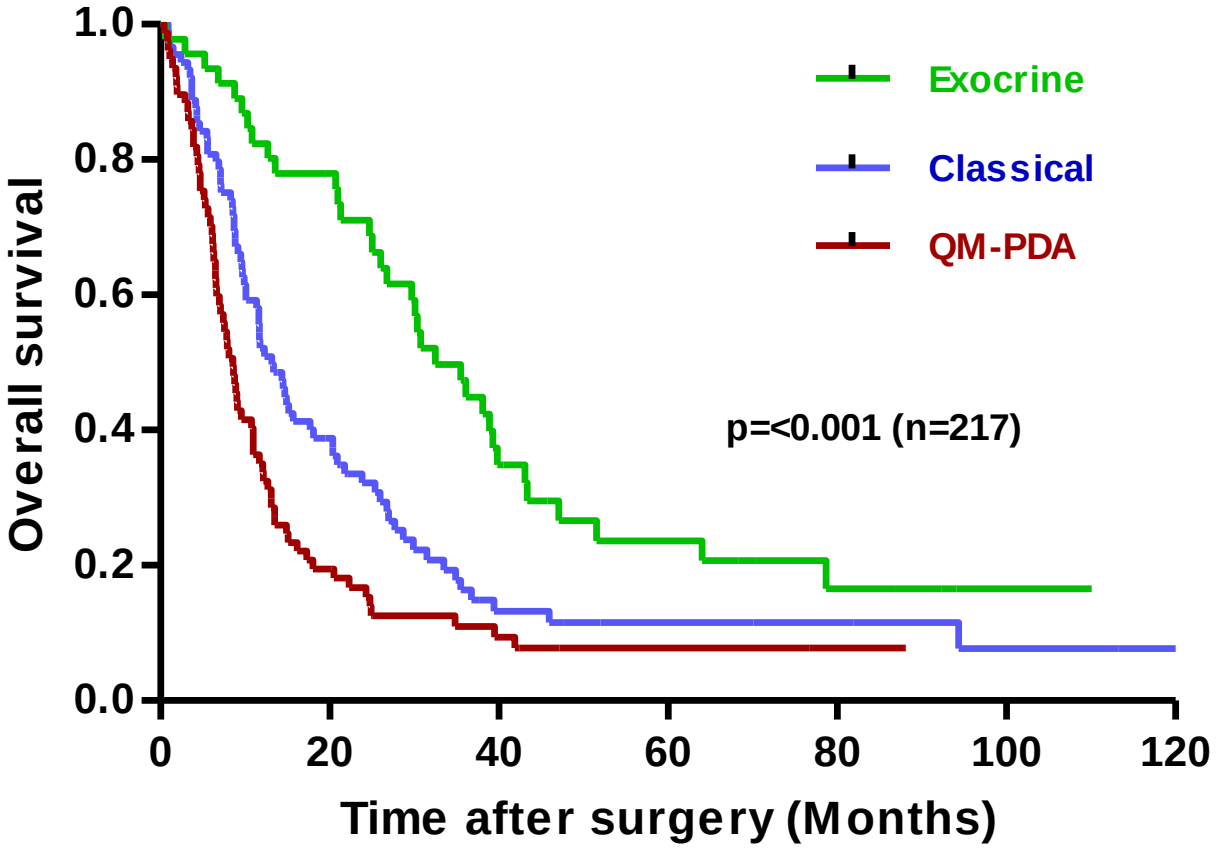


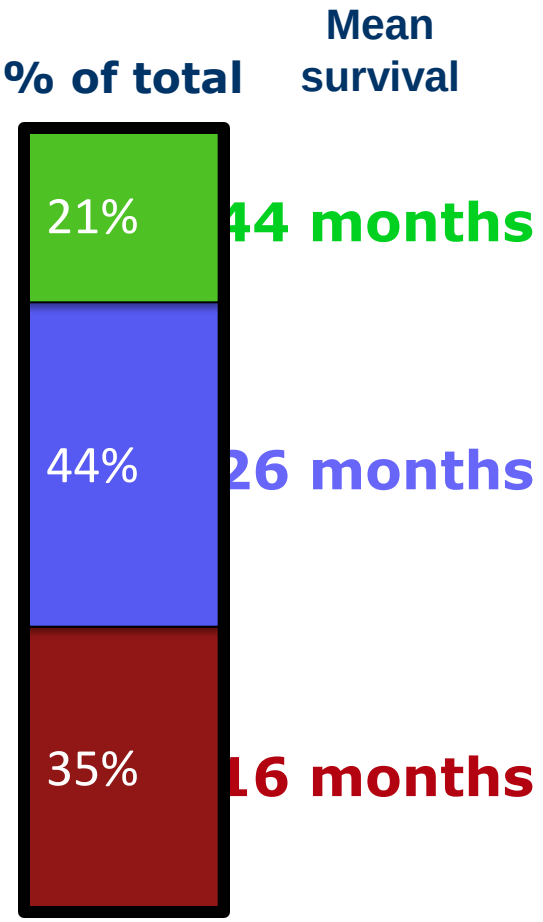


Two ABs stratify PDAC



PDAC Subtype Differences in OS





(patients with recectable tumors)

Current Predictive Tissue Tests in Oncology

Marker	Tumor Disease	Tech 1	Tech 2
ER	Breast Cancer	IHC	
HER2	Breast Cancer	IHC	FISH/CISH
HER2	Gastric Cancer	IHC	FISH/CISH
EGFR	NSCLC	Seq	
ALK	NSCLC	*IHC*	FISH/CISH
RAS	CRC	Seq	
BRAF	Melanoma	Seq	
KIT	GIST	Seq	

Currently predictive tumor markers are 50% sequencing-based and 50 % histology-based

Circulating Tumor Cells (CTCs)

- Need **enrichment**; enrichment is problematic
  - Different enrichment procedures (e.g. Adnatest, CellSearch®, OnkoQuick®)
  - Not standardized (variation ~50%)
  - High effort; prone to contamination
  - Limited applicability (e.g. EpCAM selection in CellSearch®); tumor cell enrichment?
  - Maximal enrichment efforts expensive and more invasive (e.g. GILUPI, Epic Sciences)

Current **enrichment procedures are not standardized** and show **significant variation**; **future standardisation** as required for clinical test is **questionable**

CTC – Clinical Applications?

- Not useful for any primary diagnostic procedure
  - To insensitive and variable
  - Many unknown issues
  - Too much effort (costs?)
- In vivo treatment testing? Possible, but…
  - So far insufficient success rate
  - Procedure too time consuming and labour intensive
  - No standardisation
- NIH CTC working group (CWG; preanalytic and analytical variables standardisation)

Blood-derived Nucleic Acids Analyses Research Applications

- Methods development
- Comparative testing in clinical trials
  - Tumor tissue analyses
  - Imaging (tumor load)

Limited translational research potential

But: Predictive Diagnostics remains Methodically Complex (e.g. ALK)

NGS-Report
(AmpliSeq Cancer Hotspot Panel V2)

Material
Internes Blockmaterial R-4775/13

Klinische Angaben
Bitte um EGFR- und ALK-Analyse

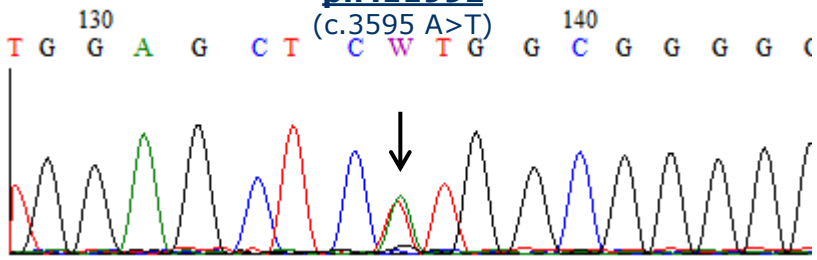
Befund
Am morphologisch gesicherten und angereicherten Tumorgewebe (50 % Tumorzellgehalt) wurde eine gezielte Mutationsanalyse mittels der Next Generation Sequenzierungstechnologie (PGM; ION TORRENT) unter der Verwendung des AmpliCancer Panels V2 (207 Amplikons; u.a. EGFR Exone 18 - 21) durchgeführt.

Hierbei wurde die Punktmutation p.M1199L mit einer Allelfrequenz von 50 % bei einer Amplikonabdeckung (Coverage) von 1012 im Exon 23 von ALK nachgewiesen.
Diese Missense-Mutation ist bisher nicht beschrieben worden. Somit liegen keine spezifischen Informationen zum ALK-Aktivierungsstatus bzw. zur TKI-Responsivität vor.

In den untersuchten Sequenzbereichen von EGFR konnte keine Mutationen detektiert werden.
Der Status aller anderen untersuchten Genabschnitte ist in unserer Datenbank hinterlegt und kann bei Bedarf (z.B. Studienkontext) angefordert werden.

Validierung der p.M1199L Mutation
(Sanger-Sequenzierung ALK Exon 23)

p.M1199L
(c.3595 A>T)

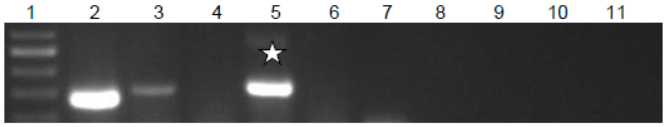


RT-PCR Analyse der EML4-ALK Fusionstranskripten
(Varianten 1 - 3)

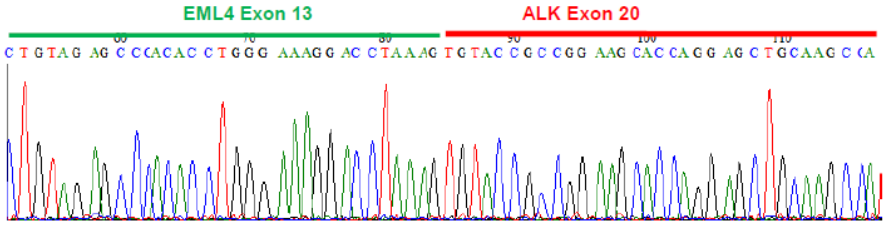
Produktgrößen
3'ALK RT-PCR: 150 bp
V1 (E13;A20) RT-PCR: 150 bp
V2 (E20;A20) RT-PCR: 138 bp
V3 (E6a/b;A20) RT-PCR: 128/161 bp
Extraktionskontrolle GAPDH: 135 bp

Nr. 1: Marker		
Nr. 2: R-4775/13	Extraktionskontrolle GAPDH	Positiv
Nr. 3: R-4775/13	**3'ALK RT-PCR**	**Positiv**
Nr. 4: R-4775/13	**V 3 (E6a/b;A20) RT-PCR**	**Negativ**
Nr. 5: R-4775/13	**V 1 (E13;A20) RT-PCR**	**Positiv**
Nr. 6: R-4775/13	**V 2 (E20;A20) RT-PCR**	**Negativ**
Nr. 7: NTC	Mix GAPDH	Negativ
Nr. 8: NTC	Mix 3'Alk	Negativ
Nr. 9: NTC	Mix E6a/b;A20	Negativ
Nr. 10: NTC	Mix E13;A20	Negativ
Nr. 11: NTC	Mix E20;A20	Negativ

*NTC = „no template control"



Sequenz des V 1 (E13;A20) RT-PCR-Produktes (Nr. 5)



Beurteilung:
Nachweis einer EML4-ALK Translokation mittels FISH sowie translokationsspezifischer RT-PCR (E13:A20) mit Bruchpunktsequenzierung (Sanger) und Nachweis der ALK-Überexpression mittels RT-PCR und Immunhistologie (D5F3, Ventana). Zusätzlich Nachweis einer M1199L Punktmutation in Exon 23 des ALK-Gens mittels NGS (PGM, Ion Torrent) und Sanger-Sequenzierung (Allelfrequenz 50%, Coverage 1012)
Die Voraussetzungen für eine Crizotinib-Therapie sind gegeben.

Clinical Improvement by NGS

- Provides oncologists with all necessary information for upfront therapy planning (clinical wish)
    - Patient information
    - Modifies therapy planning in first line
- Relevant additional information:
    - E.g. BRAF-mutations in CRC (not otherwise tested but invalidates EGFR inhibition)
    - Therapy planning in diagnostically unclear tumors (CUP)
    - Potential for targeted trials

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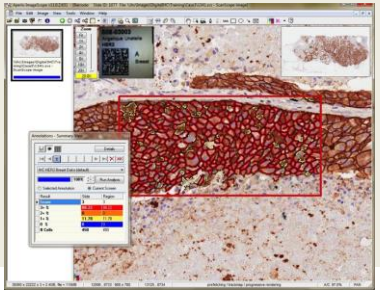
Relevant additional Innovations by Panel-NGS

- Provides patients with access clinical trials
- Essential component of CCCs (Umbrella-concepts)
- Basis for registries (monitoring; improvement of diagnostics and therapy, comparison of centers, epidemiology etc.)
- Basis for bedside-bench research improving diagnostic output

IHC/ISH automated Assessment

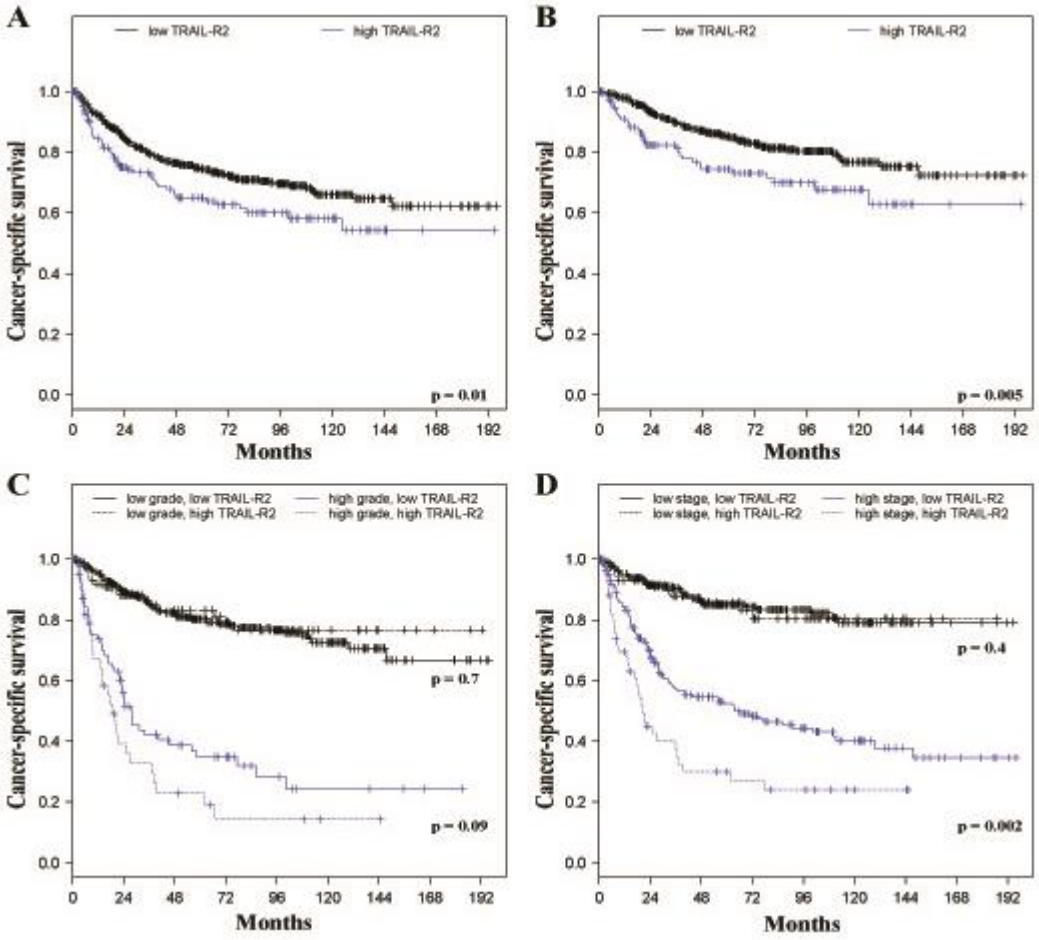
- Specified technology, work flow, and collective
- Work flows are up to it
- High pressure to provide quantitative data
- Reliable quantitative data can be produced
- Marriage of VM and image analysis
- Parallel processing

- Requires highly elaborate segmentation programs
- Needs tedious adjustment to every single test
- Additional standard incubation
- Only stepwise (testwise) implementation possible



Nevertheless, this is the proof of principle!

Correlative Data Analyses



Macher-Goeppinger et al., Clin Cancer Res 15 (2009) 650-9

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Why Panel-Sequencing in Molecular Pathology Diagnostics?

- Methodical Reasons
- Clinical Reasons
- Necessary Innovation

Some Open Questions

Which cellular source is responsible for ctDNA?

necrotic, apoptotic, or vital cells?

Which tumor compartiment is represented by ctDNA and CTCs? To which extent?

Primary? metastases? Cancer stem cells? Or none?

How is the result influenced by real world parameters

time of blood draw, source of blood draw, decay processes, interference by medications etc.

How can the results be attributed to a given tumor?

premalignant condition/carcinogenic field/secondary malignancy

How can technical/methodical questions be solved

distinguish unreliable results from true wt cases? Insufficient sensitivity? Contamination? Short t1/2?

How can the isolation procedure be standardized?

CTC-definition and isolation; ctDNA isolation