Gene Screening for Lung Cancer

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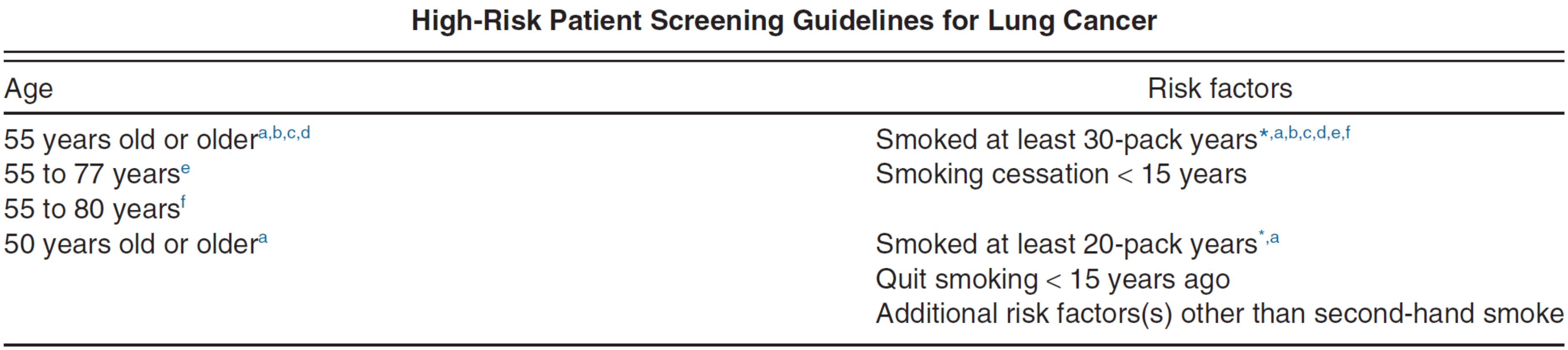
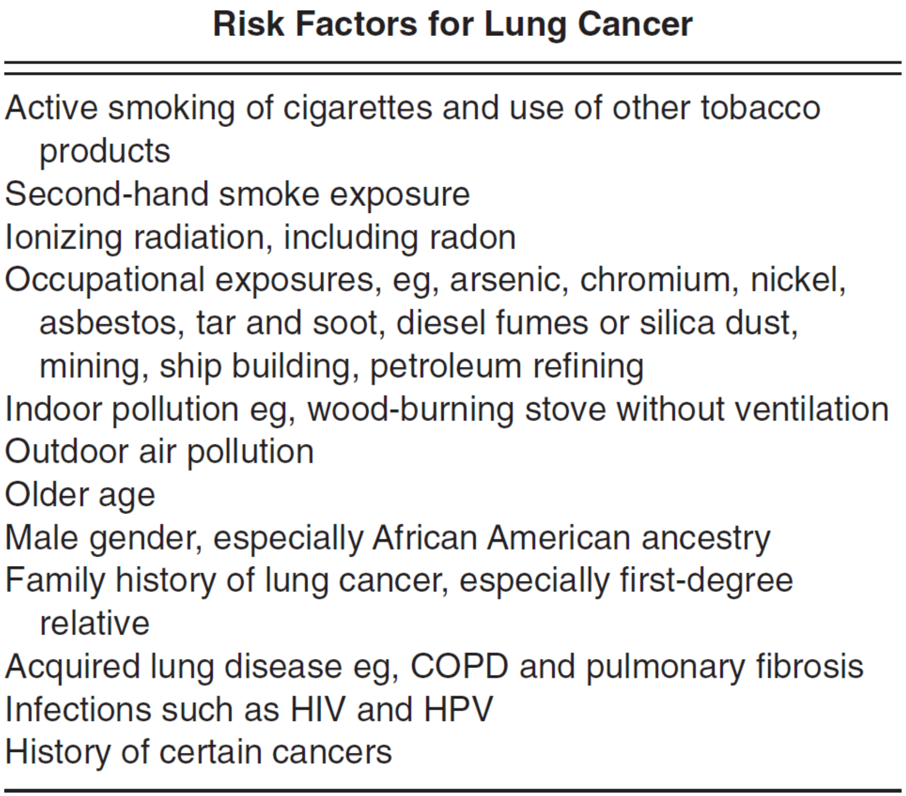
High-risk patient screening guidelines for lung cancer



High-Risk Patient Screening Guidelines for Lung Cancer

Age	Risk factors
55 years old or older[a,b,c,d]	Smoked at least 30-pack years[*,a,b,c,d,e,f]
55 to 77 years[e]	Smoking cessation < 15 years
55 to 80 years[f]	
50 years old or older[a]	Smoked at least 20-pack years[*,a]
	Quit smoking < 15 years ago
	Additional risk factors(s) other than second-hand smoke

[a] National Comprehensive Cancer Network.[41]
[b] American Cancer Society.[42]
[c] American Society of Clinical Oncologists.[43]
[d] American College of Chest physicians.[44]
[e] Medicare & Medicaid services in 2017.[45]
[f] US Preventive Services Task Force, 2014.[46]

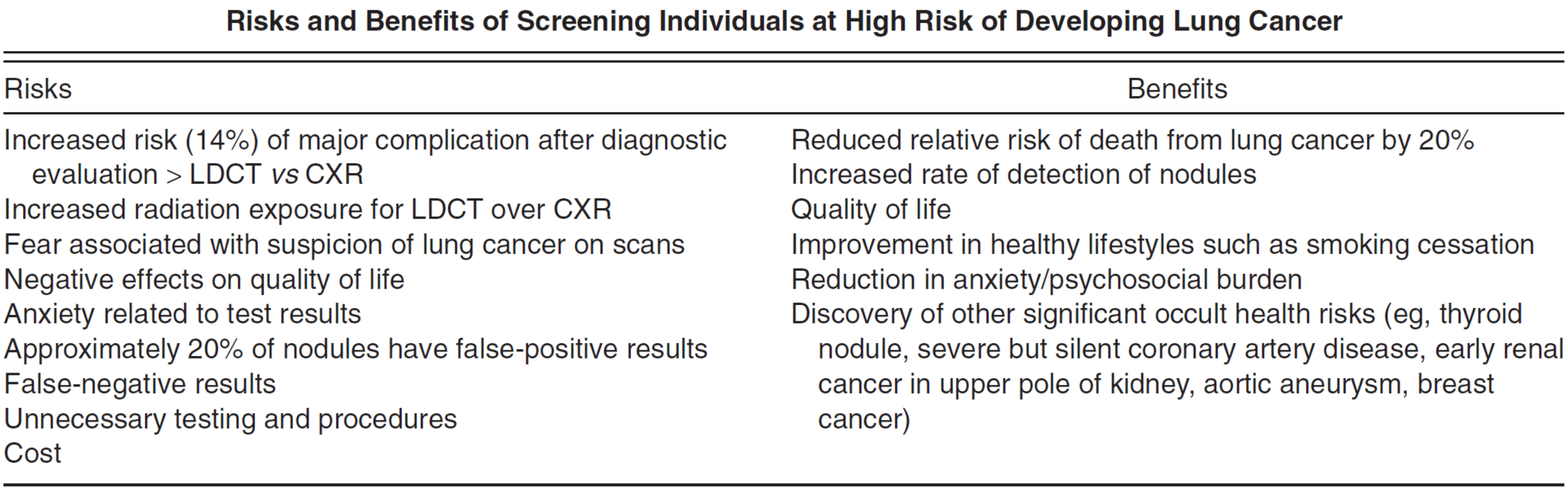


Risk Factors for Lung Cancer

Active smoking of cigarettes and use of other tobacco products
Second-hand smoke exposure
Ionizing radiation, including radon
Occupational exposures, eg, arsenic, chromium, nickel, asbestos, tar and soot, diesel fumes or silica dust, mining, ship building, petroleum refining
Indoor pollution eg, wood-burning stove without ventilation
Outdoor air pollution
Older age
Male gender, especially African American ancestry
Family history of lung cancer, especially first-degree relative
Acquired lung disease eg, COPD and pulmonary fibrosis
Infections such as HIV and HPV
History of certain cancers

Screening and early detection of lung cancer

- Sputum cytology
- Chest X-ray
- Chest CT
- Low-dose CT
- PET/CT



Risks and Benefits of Screening Individuals at High Risk of Developing Lung Cancer

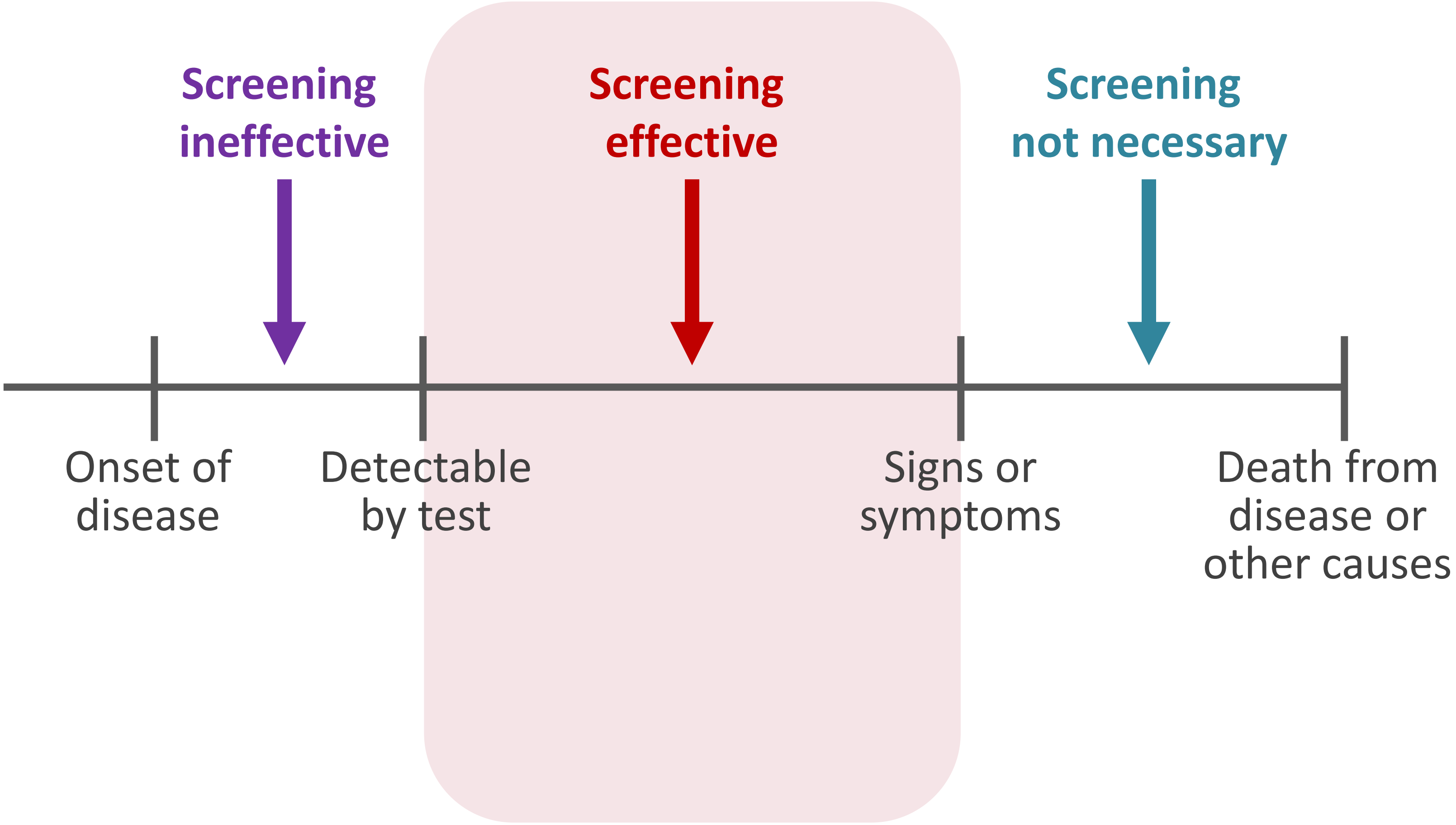
Risks	Benefits
Increased risk (14%) of major complication after diagnostic evaluation > LDCT *vs* CXR	Reduced relative risk of death from lung cancer by 20%
Increased radiation exposure for LDCT over CXR	Increased rate of detection of nodules
Fear associated with suspicion of lung cancer on scans	Quality of life
Negative effects on quality of life	Improvement in healthy lifestyles such as smoking cessation
Anxiety related to test results	Reduction in anxiety/psychosocial burden
Approximately 20% of nodules have false-positive results	Discovery of other significant occult health risks (eg, thyroid nodule, severe but silent coronary artery disease, early renal cancer in upper pole of kidney, aortic aneurysm, breast cancer)
False-negative results	
Unnecessary testing and procedures	
Cost	

Biomarker for early detection may developed for:

- Identification of at-risk individuals, who may be screened with LDCT after a positive marker test.

- After the first LDCT screen shows a solid lesion, a biomarker may be serve as a diagnostic test for malignancy.

- After the first LDCT screen shows a ground glass lesion, determine whether the lesion has a high or low chance of becoming malignant.


Screening ineffective
Screening effective
Screening not necessary
Onset of disease
Detectable by test
Signs or symptoms
Death from disease or other causes

Molecular markers for lung cancer screening

Specimen

- Sputum
- **Blood**
- Urine
- Exhaled breath

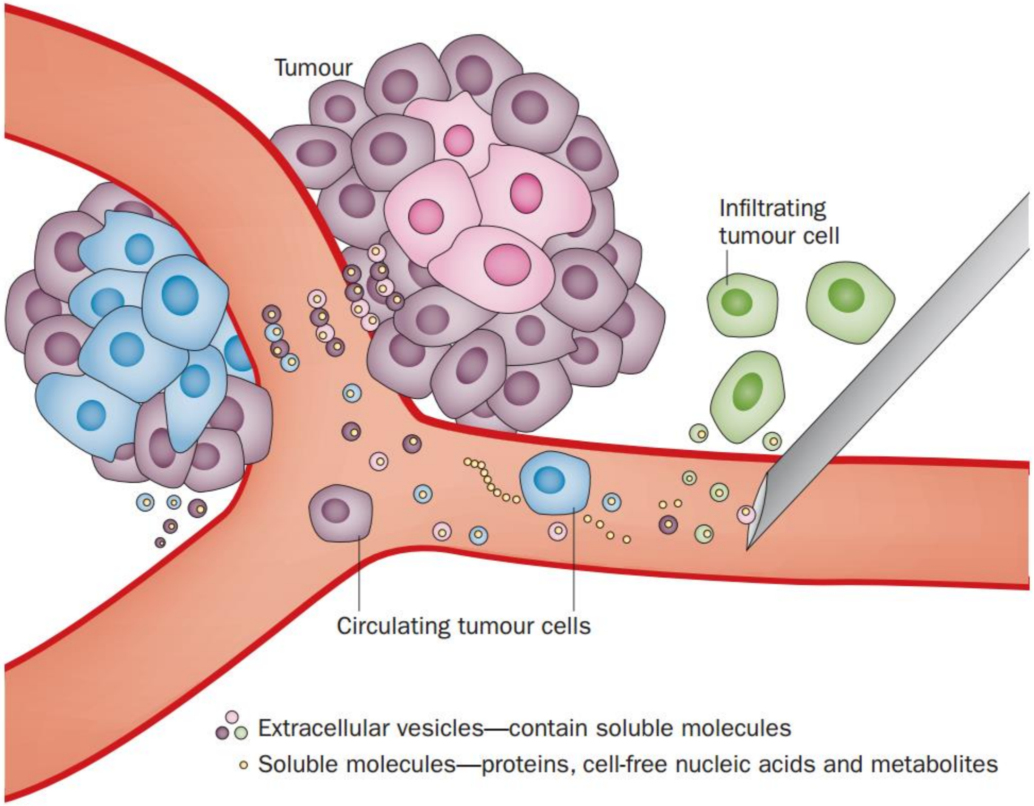
Markers

- Circulating tumor DNA
- Cell-free DNA amount
- **DNA mutation**
- DNA methylation
- miRNA
- Autoantibodies
- Proteomic profiles
- Volatile organic compounds

Blood-based tumor diagnostics

(“Liquid Biopsy”)

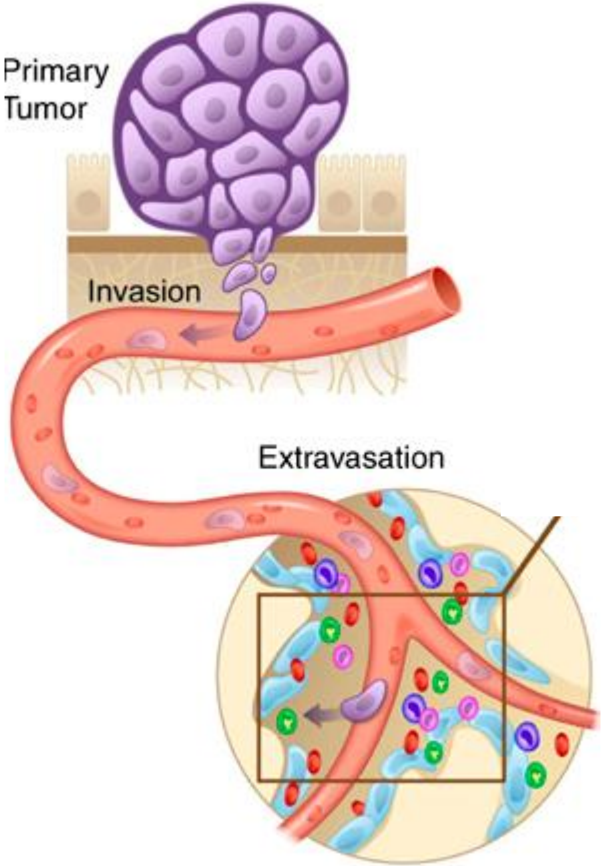
- Circulating tumor cells (CTC)
- Circulating cell-free nucleic acids
  - circulating tumor DNA (ctDNA)
  - circulating tumor RNA (ctRNA)
  - microRNA (miRNA)
- Nucleosome
- Exosome

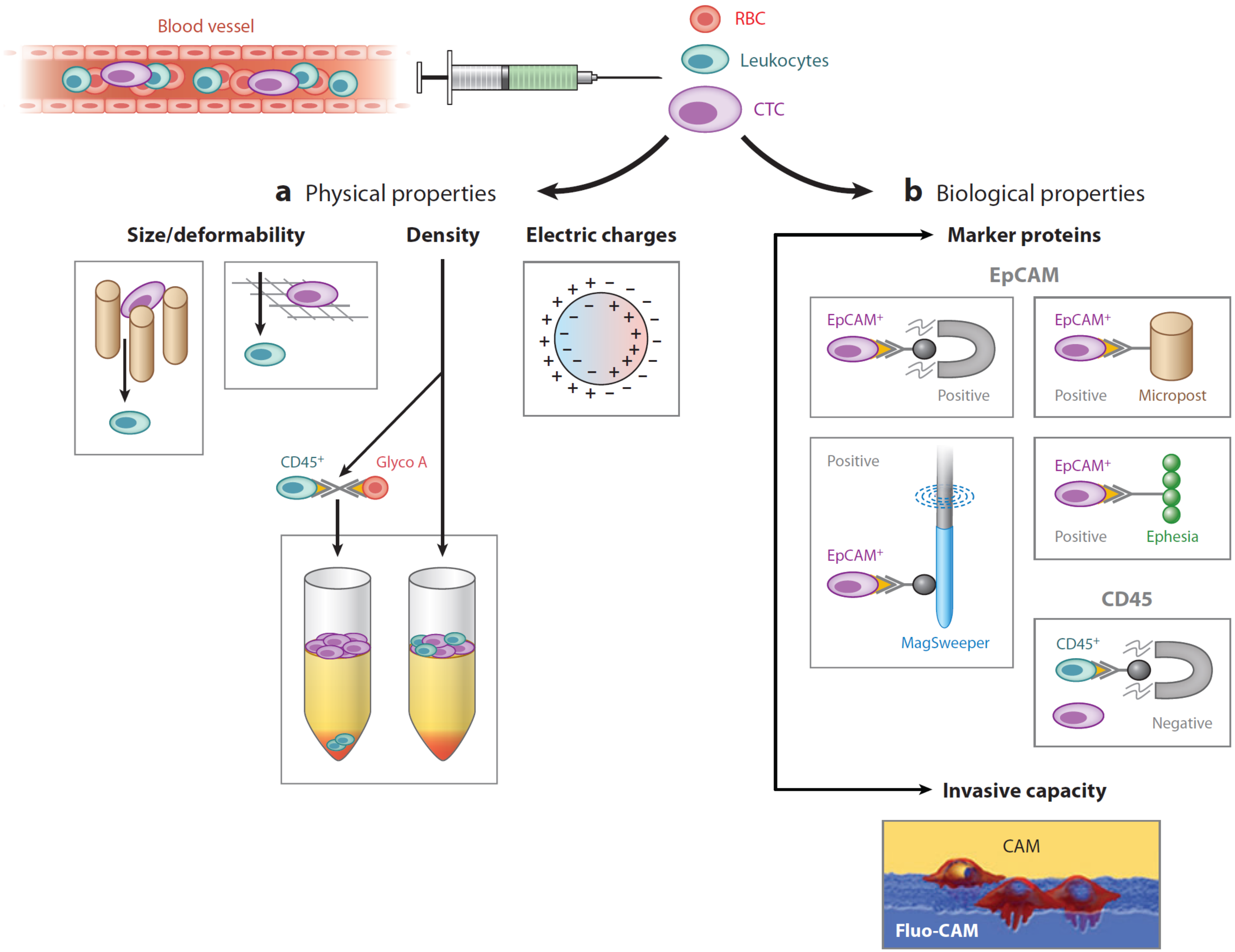


Circulating Tumor Cells (CTC)

- Tumor cells shed into the blood stream
  - disseminated cancer cells
  - cancer stem cells
  - bystander cells

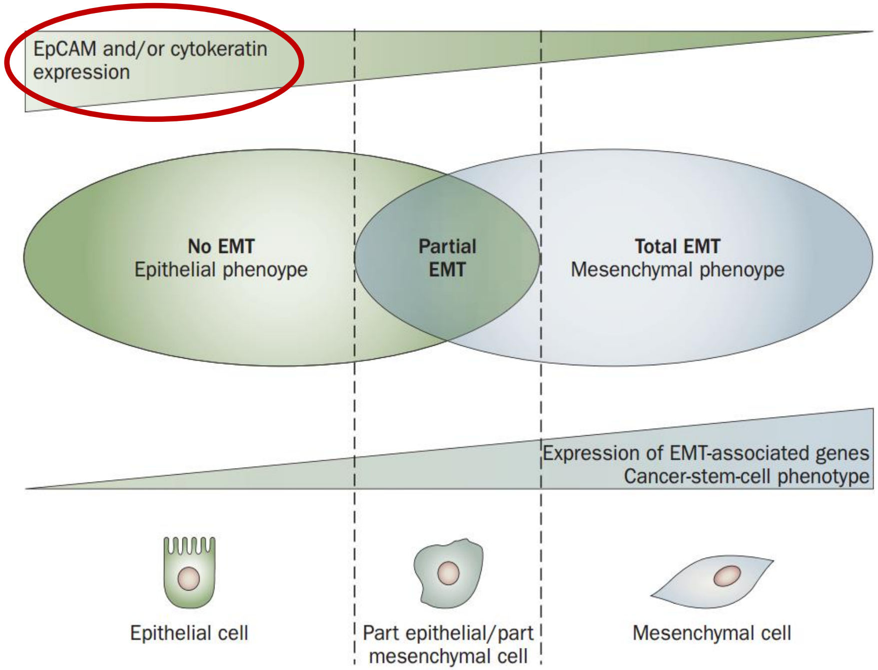
- 1 per 10^6~10^7 leukocytes
- Average 100 per 1 mL blood
  - Conventional methods may undercount the number of CTCs

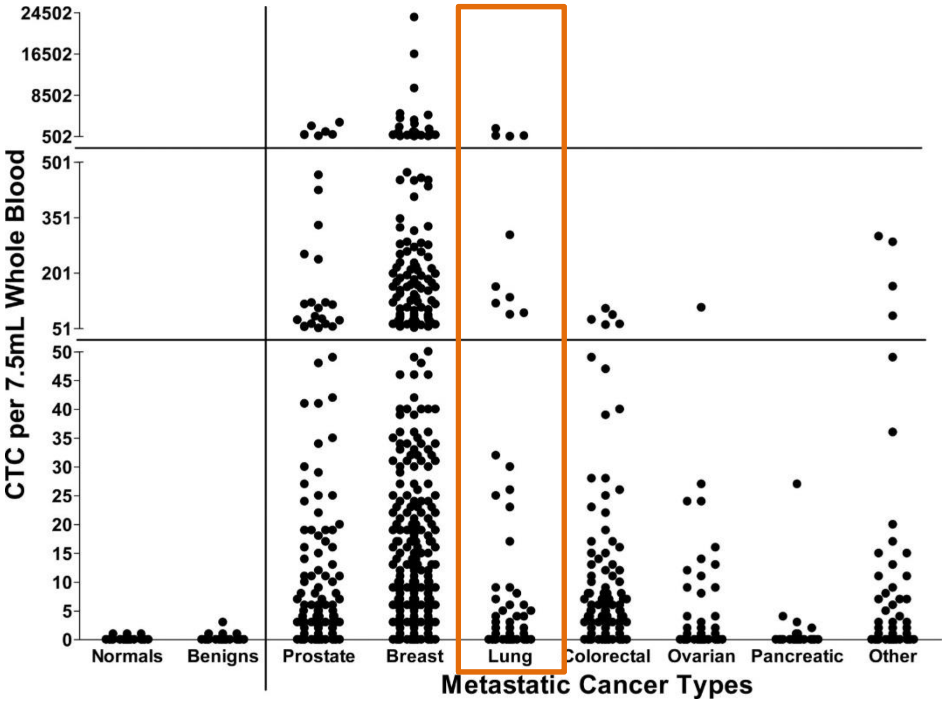



Blood vessel
RBC
Leukocytes
CTC
a Physical properties
b Biological properties
Size/deformability
Density
Electric charges
CD45+
Glyco A
Marker proteins
EpCAM
EpCAM+
Positive
EpCAM+
Positive
Micropost
Positive
EpCAM+
MagSweeper
EpCAM+
Positive
Ephesia
CD45
CD45+
Negative
Invasive capacity
CAM
Fluo-CAM

CTC - Limitations

- Only a proportion of cases have detectable CTCs
- Epithelial–mesenchymal transition (EMT)
- Positive rates differ among different tumors





Allard et al. Clin Cancer Res. 2004

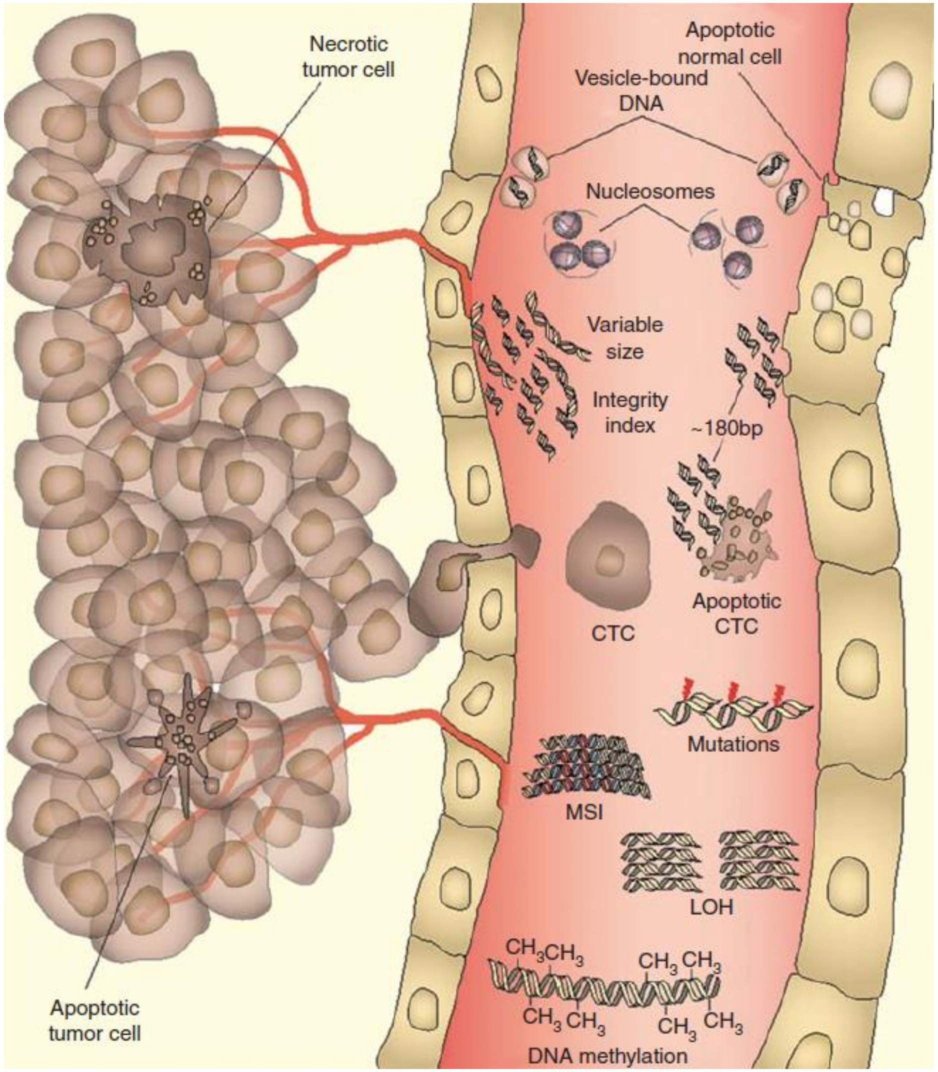
Circulating cell-free DNAs

- Various sources
  - Normal cells

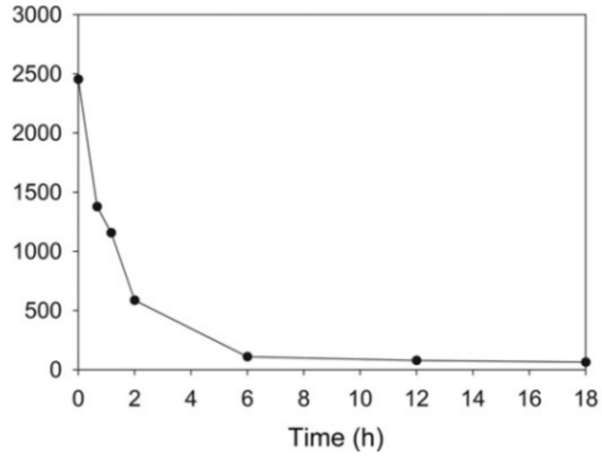
  - Tumor necrosis
  - Tumor apoptosis
  - Circulating tumor cells

- Variable amount
  - <30 ng ~ > 1000 ng/5mL

- Variable fraction
  - ctDNA: <0.01% ~ >90%

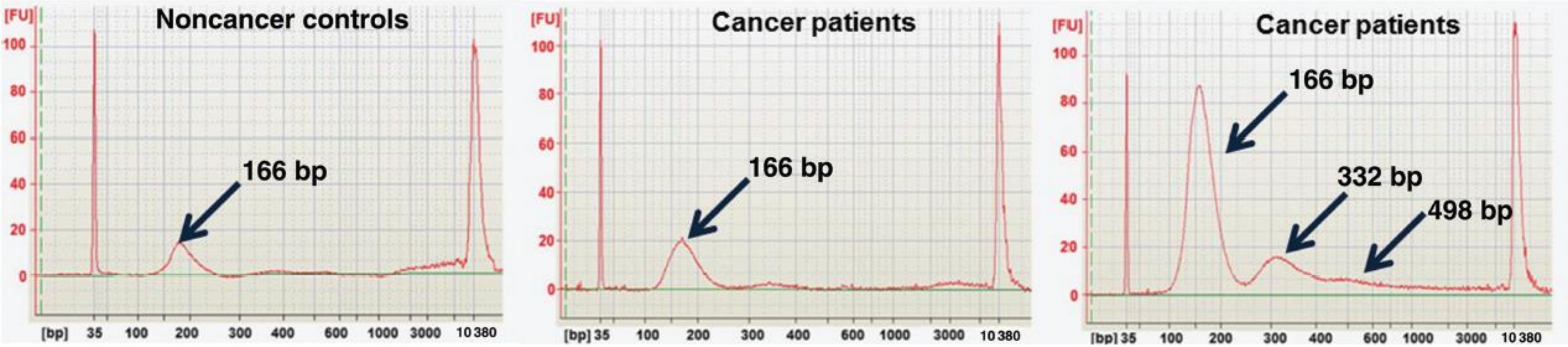


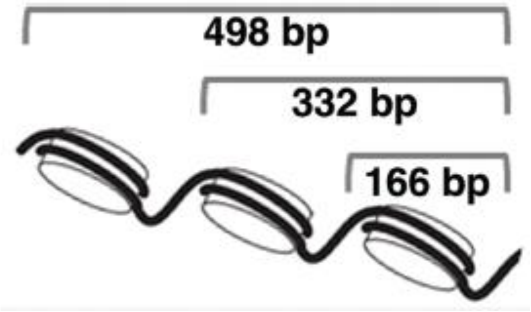
Collection of cell-free DNA



- Short half life (15 min ~ several hours) due to plasma DNases
- Blood can be sampled in ETDA tubes but the plasma has to isolated and stored at -80^{o}C within one hour of collection
- **Preservative tubes**
  - 4~7 days at room temperature
  - Cell-free DNA BCT (Streck), PreAnalytix (PAXgene), Roche, NORGEN, etc.
- **Plasma vs. serum**
  - DNA concentrations: serum > plasma
  - Serum contains large amount of leukocyte-derived DNAs
  - EDTA has some inhibitory effect on DNase

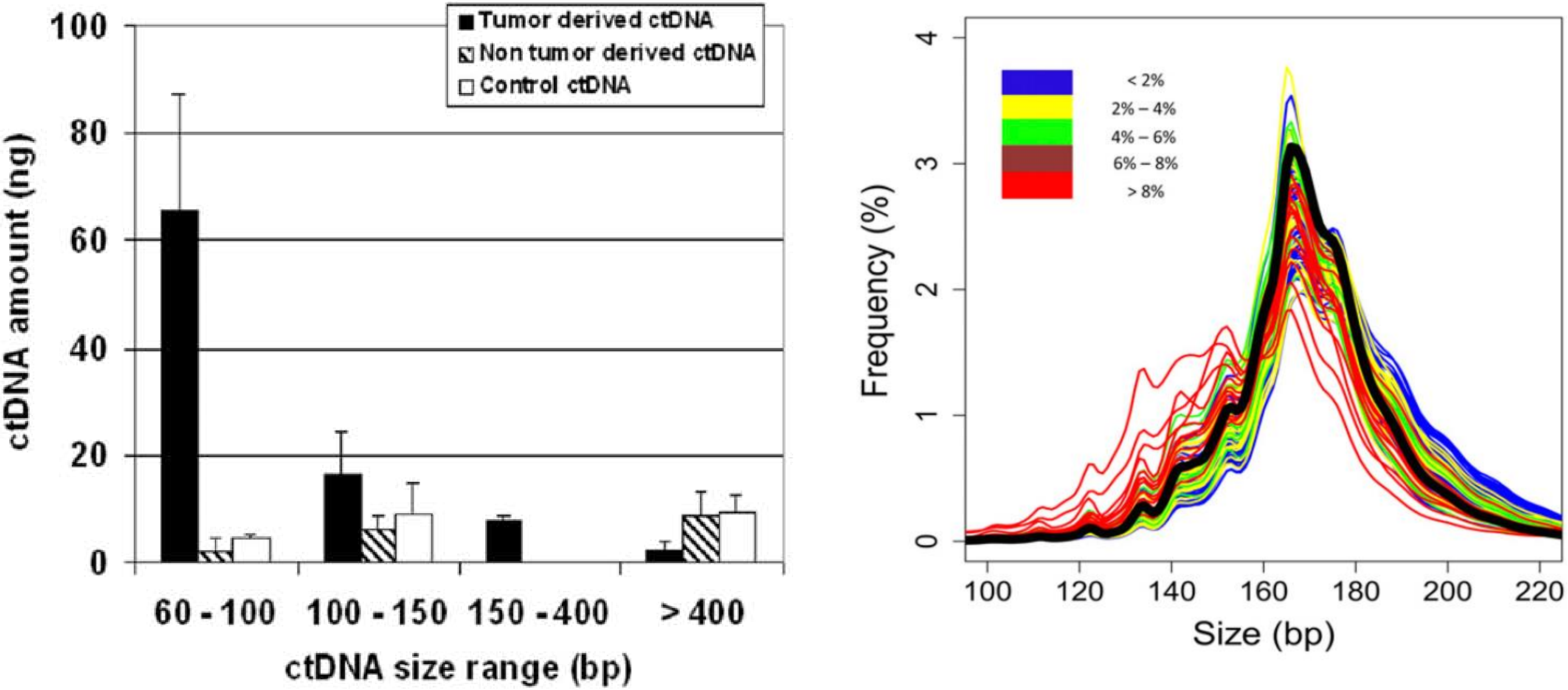
Size distribution of ctDNA





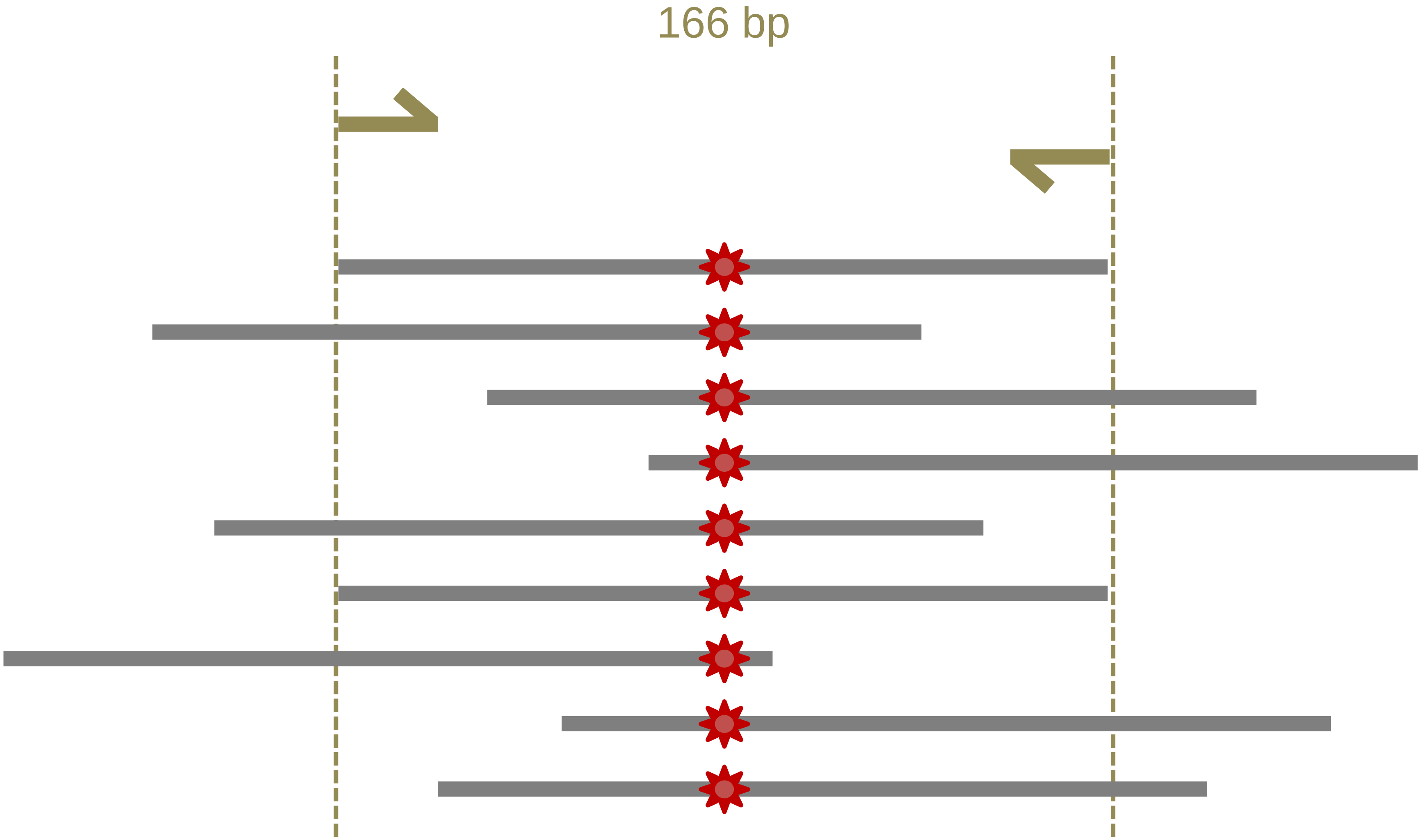
Heitzer et al. Clin Chem. 2015

High fragmentation of ctDNA

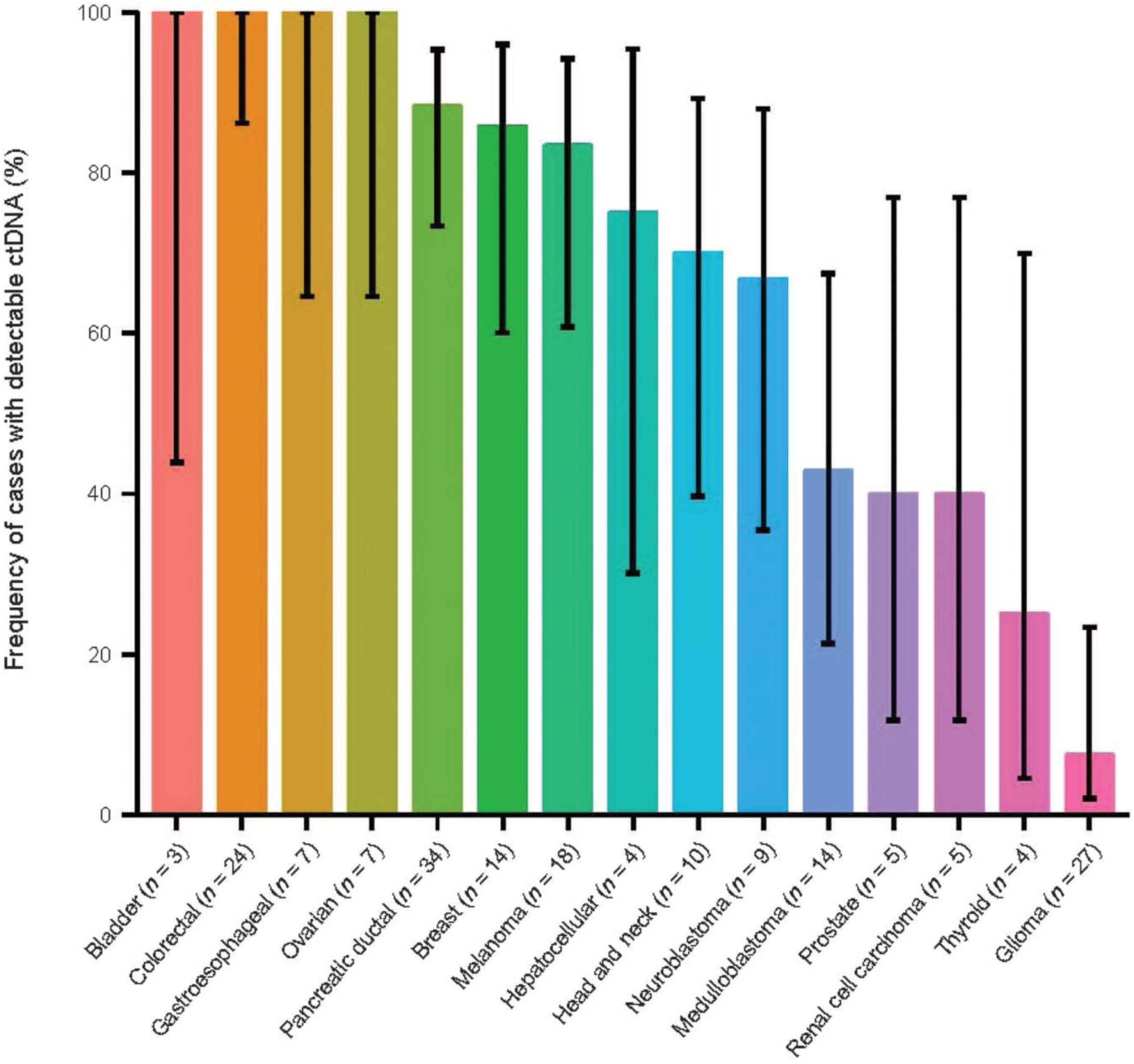


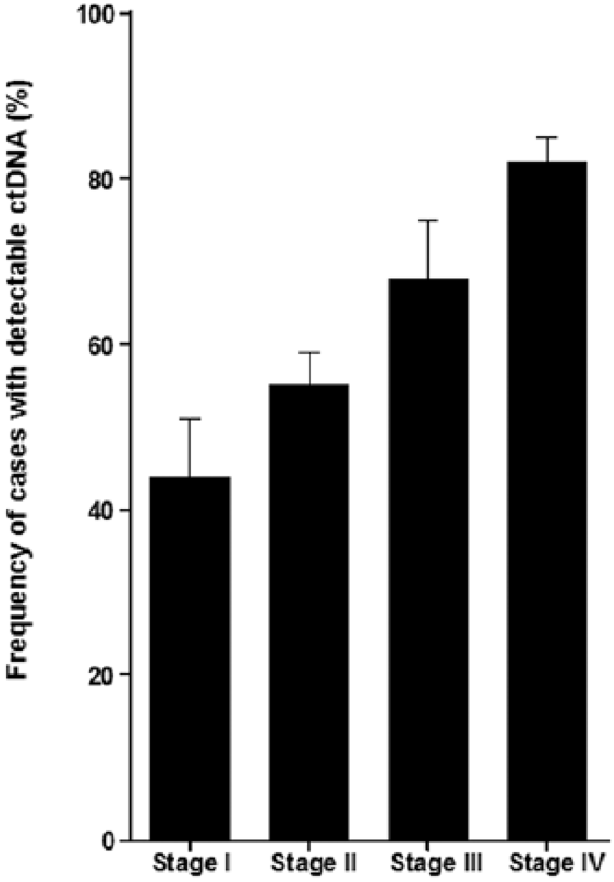
Mouliere et al. PLOS One 2011; Jiang et al. PNAS 2015

Location of target mutation



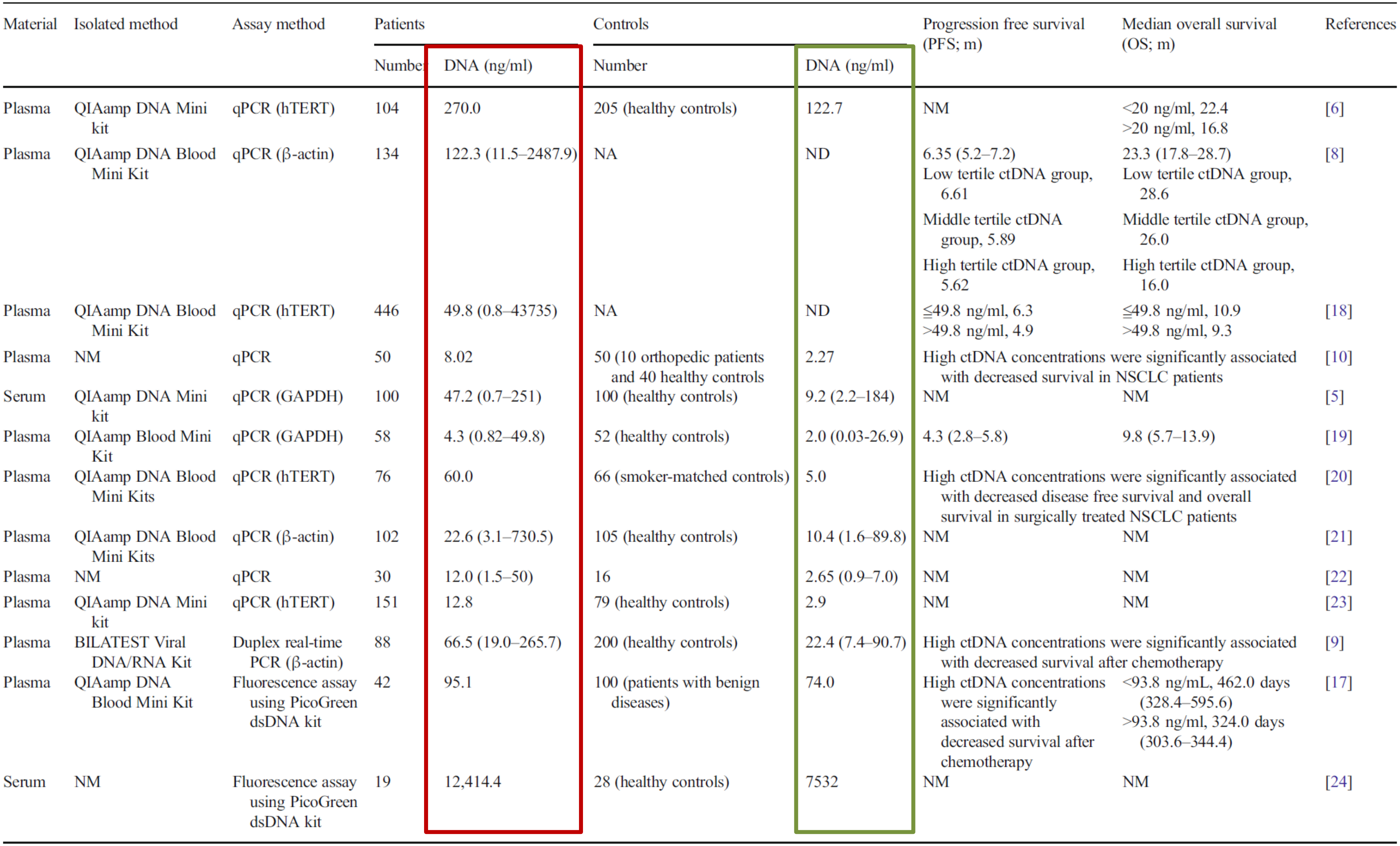
Detection rates of ctDNA





Bettegowda et al. Science Translational Med. 2014

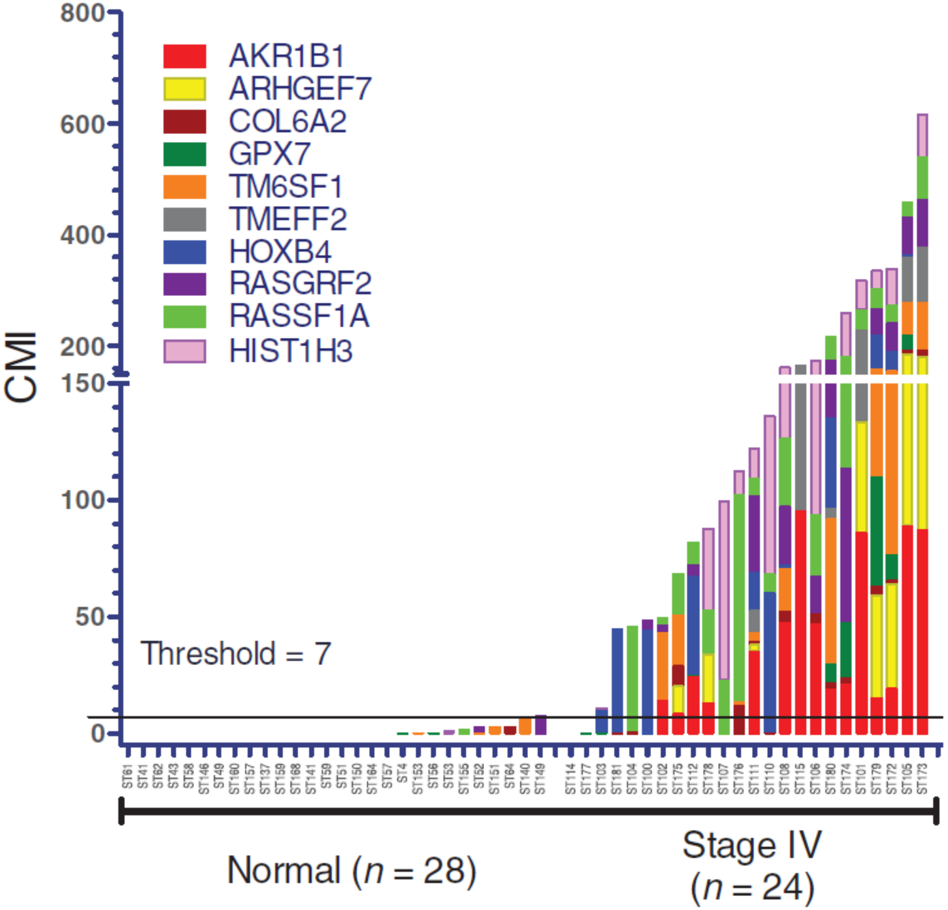
Quantitative estimates of plasma/serum of cell-free DNA

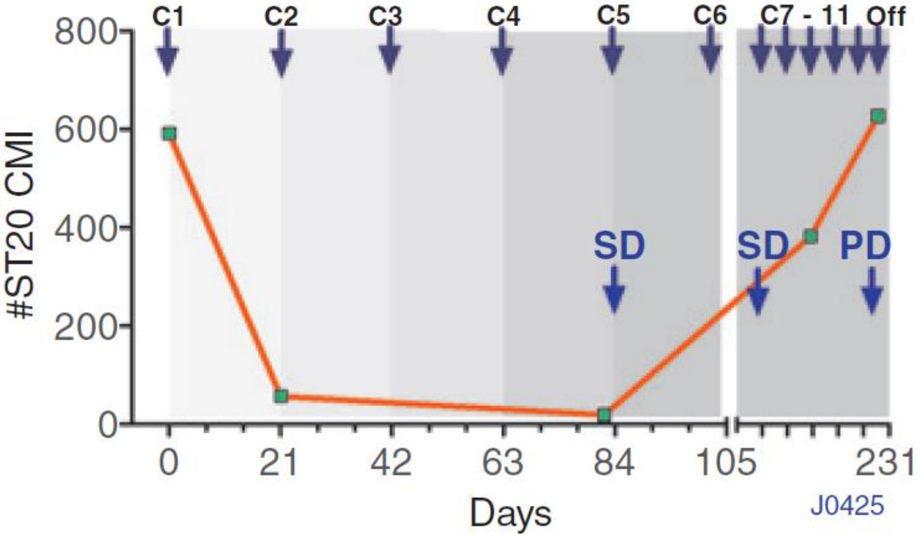


Material	Isolated method	Assay method	Patients		Controls		Progression free survival (PFS; m)	Median overall survival (OS; m)	References
			Numbe	DNA (ng/ml)	Number	DNA (ng/ml)			
Plasma	QIAamp DNA Mini kit	qPCR (hTERT)	104	270.0	205 (healthy controls)	122.7	NM	<20 ng/ml, 22.4 >20 ng/ml, 16.8	[6]
Plasma	QIAamp DNA Blood Mini Kit	qPCR (β-actin)	134	122.3 (11.5–2487.9)	NA	ND	6.35 (5.2–7.2) Low tertile ctDNA group, 6.61 Middle tertile ctDNA group, 5.89 High tertile ctDNA group, 5.62	23.3 (17.8–28.7) Low tertile ctDNA group, 28.6 Middle tertile ctDNA group, 26.0 High tertile ctDNA group, 16.0	[8]
Plasma	QIAamp DNA Blood Mini Kit	qPCR (hTERT)	446	49.8 (0.8–43735)	NA	ND	≤49.8 ng/ml, 6.3 >49.8 ng/ml, 4.9	≤49.8 ng/ml, 10.9 >49.8 ng/ml, 9.3	[18]
Plasma	NM	qPCR	50	8.02	50 (10 orthopedic patients and 40 healthy controls	2.27	High ctDNA concentrations were significantly associated with decreased survival in NSCLC patients		[10]
Serum	QIAamp DNA Mini kit	qPCR (GAPDH)	100	47.2 (0.7–251)	100 (healthy controls)	9.2 (2.2–184)	NM	NM	[5]
Plasma	QIAamp Blood Mini Kit	qPCR (GAPDH)	58	4.3 (0.82–49.8)	52 (healthy controls)	2.0 (0.03-26.9)	4.3 (2.8–5.8)	9.8 (5.7–13.9)	[19]
Plasma	QIAamp DNA Blood Mini Kits	qPCR (hTERT)	76	60.0	66 (smoker-matched controls)	5.0	High ctDNA concentrations were significantly associated with decreased disease free survival and overall survival in surgically treated NSCLC patients		[20]
Plasma	QIAamp DNA Blood Mini Kits	qPCR (β-actin)	102	22.6 (3.1–730.5)	105 (healthy controls)	10.4 (1.6–89.8)	NM	NM	[21]
Plasma	NM	qPCR	30	12.0 (1.5–50)	16	2.65 (0.9–7.0)	NM	NM	[22]
Plasma	QIAamp DNA Mini kit	qPCR (hTERT)	151	12.8	79 (healthy controls)	2.9	NM	NM	[23]
Plasma	BILATEST Viral DNA/RNA Kit	Duplex real-time PCR (β-actin)	88	66.5 (19.0–265.7)	200 (healthy controls)	22.4 (7.4–90.7)	High ctDNA concentrations were significantly associated with decreased survival after chemotherapy		[9]
Plasma	QIAamp DNA Blood Mini Kit	Fluorescence assay using PicoGreen dsDNA kit	42	95.1	100 (patients with benign diseases)	74.0	High ctDNA concentrations were significantly associated with decreased survival after chemotherapy	<93.8 ng/mL, 462.0 days (328.4–595.6) >93.8 ng/ml, 324.0 days (303.6–344.4)	[17]
Serum	NM	Fluorescence assay using PicoGreen dsDNA kit	19	12,414.4	28 (healthy controls)	7532	NM	NM	[24]

Nie et al. Tumor Biol. 2015

DNA methylation



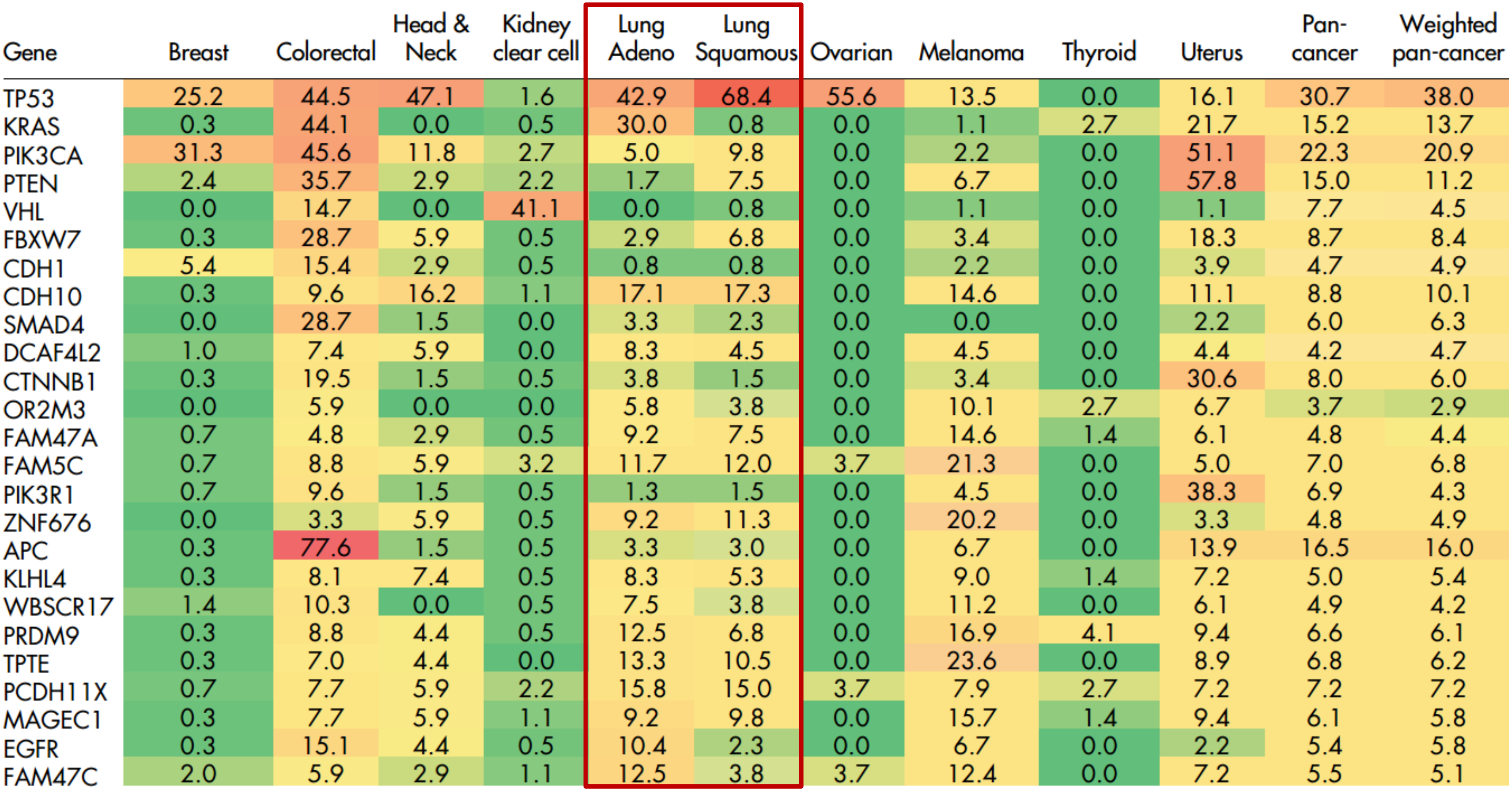


Fackler et al. Cancer Res. 2014

Material	Method	Genes analyzed	Patients		Controls		Median progression free survival		Median overall survival		Sensitivity (%)	Specificity (%)	References
			Number	Frequency (%)	Number	Frequency (%)	Methylated (m)	Unmethylated (m)	Methylated (m)	Unmethylated (m)			
Plasma	MSP	P16	67	17.9	NA	ND	31 weeks	24 weeks	53 weeks	43 weeks	NM	NM	[68]
Serum	Nested MSP	TMEFF2	316	9.2	50 (age-matched healthy controls)	0	NM	NM	NM	NM	9.2	100	[75]
Plasma	MSP	RARbeta2	52	51	26 (healthy controls)	18	NM	NM	NM	NM	70	63	[76]
Plasma	AQAMA	SHP1P2	38	92	52 (age- and sex-matched healthy controls)	38	<700 pg/ml, 5.2 >700 pg/ml, 2.6	NM	<700 pg/ml, 12.6 >700 pg/ml, 7.6	NM	89.5	90.4	[77]
Plasma	MSP	DLEC1	78	35.9	50 (healthy controls and patients with benign diseases)	2	NM	NM	NM	NM	84.4	97.8	[78]
Plasma	MSP	APC	110	47.27	50 (healthy controls and patients with benign diseases)	10.0	NM	NM	NM	NM	NM	NM	[73]
		CDH13		33.64		4.0							
		KLK10		29.09		4.0							
		DLEC1		25.45		2.0							
		RASSF1A		36.36		8.0							
		EFEMP1		21.82		6.0							
		SFRP1		23.64		4.0							
		RARbeta		20.0		6.0							
		p16		22.73		8.0							
Plasma	MSP	SFRP1	78	28.2	50 (healthy controls and patients with benign diseases)	4.0	NM	NM	NM	NM	NM	NM	[79]
Serum	qMSP (b-Actin)	DAPK	76	68.4	NA	ND	NM	NM	NM	NM	NM	NM	[80]
		MGMT		7.9									
Serum	nested MSP	APC1A	92	30.8	14 (healthy controls)	0.0	NM	NM	8.6	11.8	NM	NM	[74]
		DAPK		26.1		0.0			11.6	10.5			
		FHIT		47.3		0.0			9.7	11.3			
		p14(ARF)		30.4		0.0			12.9	9.0			
		p16		25.9		0.0			8.2	11.2			
		RARbeta		44.6		14.3			12.0	8.8			
		RASSF1A		33.3		0.0			12.0	10.5			
Serum	MSP	CHFR	308	32.4	NA	ND	NM	NM	11.50 (chemotherapy) 14.12 (EGFR TKIs)	11.21 (chemotherapy) 21.36 (EGFR TKIs)	NM	NM	[47]
Plasma	MSP	KLK10	78	38.7	50 (healthy controls and patients with benign diseases)	4.0	NM	NM	NM	NM	80.6	97.6	[81]

Nie et al. Tumor Biol. 2015

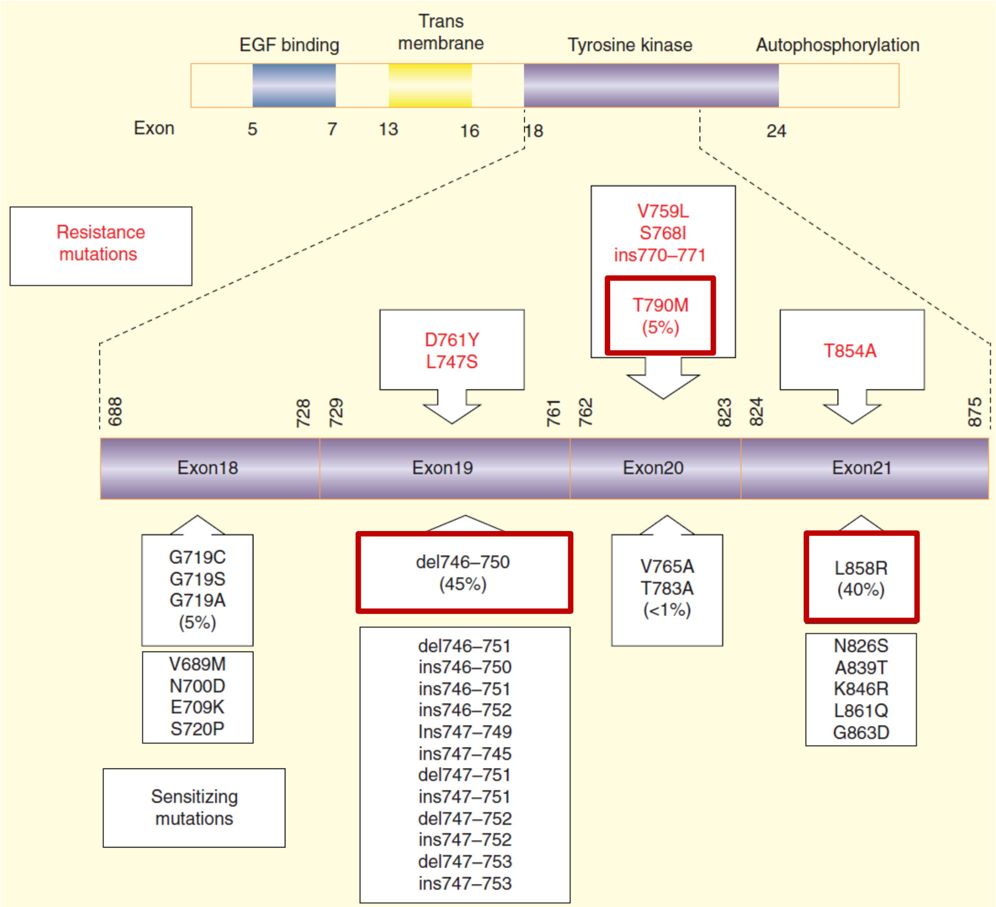
Mutation frequencies in different cancers

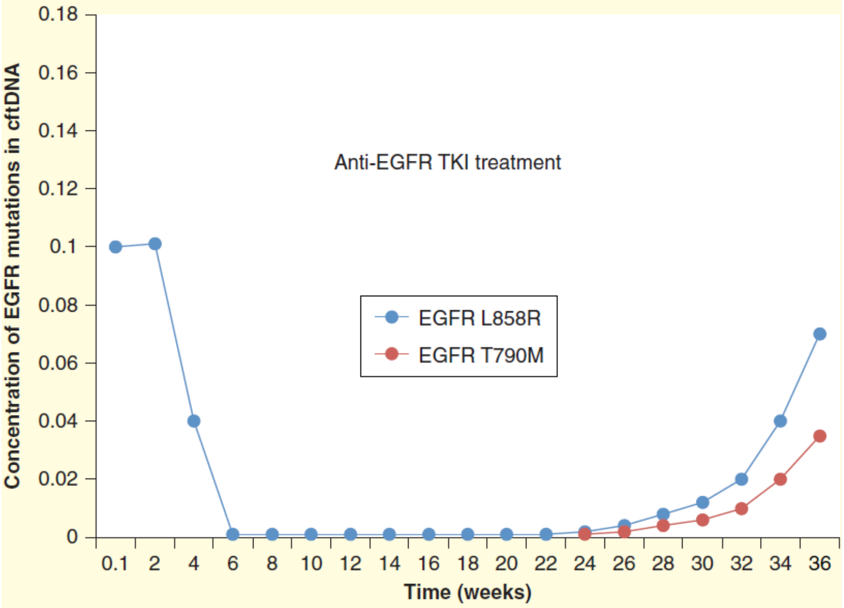


Gene	Breast	Colorectal	Head & Neck	Kidney clear cell	Lung Adeno	Lung Squamous	Ovarian	Melanoma	Thyroid	Uterus	Pan-cancer	Weighted pan-cancer
TP53	25.2	44.5	47.1	1.6	42.9	68.4	55.6	13.5	0.0	16.1	30.7	38.0
KRAS	0.3	44.1	0.0	0.5	30.0	0.8	0.0	1.1	2.7	21.7	15.2	13.7
PIK3CA	31.3	45.6	11.8	2.7	5.0	9.8	0.0	2.2	0.0	51.1	22.3	20.9
PTEN	2.4	35.7	2.9	2.2	1.7	7.5	0.0	6.7	0.0	57.8	15.0	11.2
VHL	0.0	14.7	0.0	41.1	0.0	0.8	0.0	1.1	0.0	1.1	7.7	4.5
FBXW7	0.3	28.7	5.9	0.5	2.9	6.8	0.0	3.4	0.0	18.3	8.7	8.4
CDH1	5.4	15.4	2.9	0.5	0.8	0.8	0.0	2.2	0.0	3.9	4.7	4.9
CDH10	0.3	9.6	16.2	1.1	17.1	17.3	0.0	14.6	0.0	11.1	8.8	10.1
SMAD4	0.0	28.7	1.5	0.0	3.3	2.3	0.0	0.0	0.0	2.2	6.0	6.3
DCAF4L2	1.0	7.4	5.9	0.0	8.3	4.5	0.0	4.5	0.0	4.4	4.2	4.7
CTNNB1	0.3	19.5	1.5	0.5	3.8	1.5	0.0	3.4	0.0	30.6	8.0	6.0
OR2M3	0.0	5.9	0.0	0.0	5.8	3.8	0.0	10.1	2.7	6.7	3.7	2.9
FAM47A	0.7	4.8	2.9	0.5	9.2	7.5	0.0	14.6	1.4	6.1	4.8	4.4
FAM5C	0.7	8.8	5.9	3.2	11.7	12.0	3.7	21.3	0.0	5.0	7.0	6.8
PIK3R1	0.7	9.6	1.5	0.5	1.3	1.5	0.0	4.5	0.0	38.3	6.9	4.3
ZNF676	0.0	3.3	5.9	0.5	9.2	11.3	0.0	20.2	0.0	3.3	4.8	4.9
APC	0.3	77.6	1.5	0.5	3.3	3.0	0.0	6.7	0.0	13.9	16.5	16.0
KLHL4	0.3	8.1	7.4	0.5	8.3	5.3	0.0	9.0	1.4	7.2	5.0	5.4
WBSCR17	1.4	10.3	0.0	0.5	7.5	3.8	0.0	11.2	0.0	6.1	4.9	4.2
PRDM9	0.3	8.8	4.4	0.5	12.5	6.8	0.0	16.9	4.1	9.4	6.6	6.1
TPTE	0.3	7.0	4.4	0.0	13.3	10.5	0.0	23.6	0.0	8.9	6.8	6.2
PCDH11X	0.7	7.7	5.9	2.2	15.8	15.0	3.7	7.9	2.7	7.2	7.2	7.2
MAGEC1	0.3	7.7	5.9	1.1	9.2	9.8	0.0	15.7	1.4	9.4	6.1	5.8
EGFR	0.3	15.1	4.4	0.5	10.4	2.3	0.0	6.7	0.0	2.2	5.4	5.8
FAM47C	2.0	5.9	2.9	1.1	12.5	3.8	3.7	12.4	0.0	7.2	5.5	5.1

Martinez et al. Sci Reports. 2013

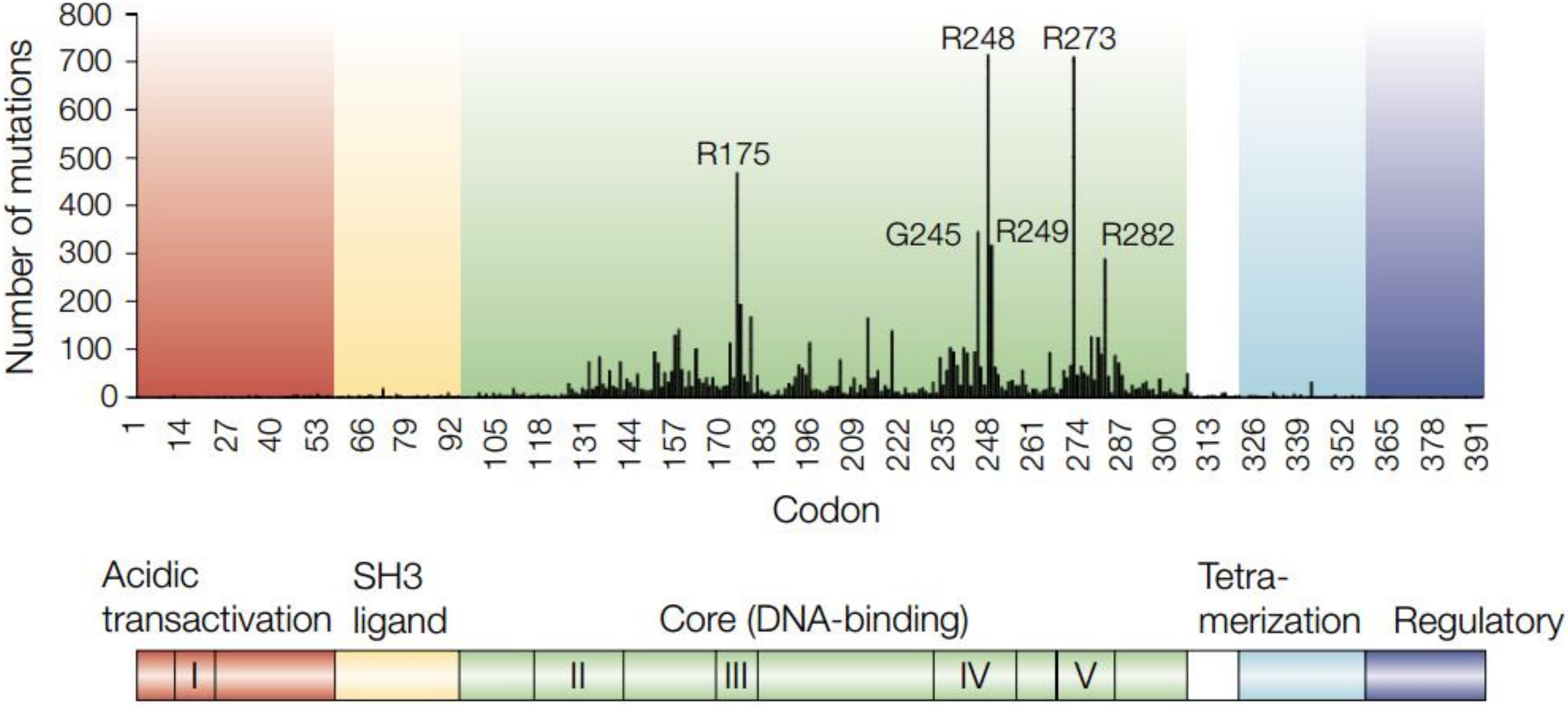
EGFR hotspot mutations in lung cancer

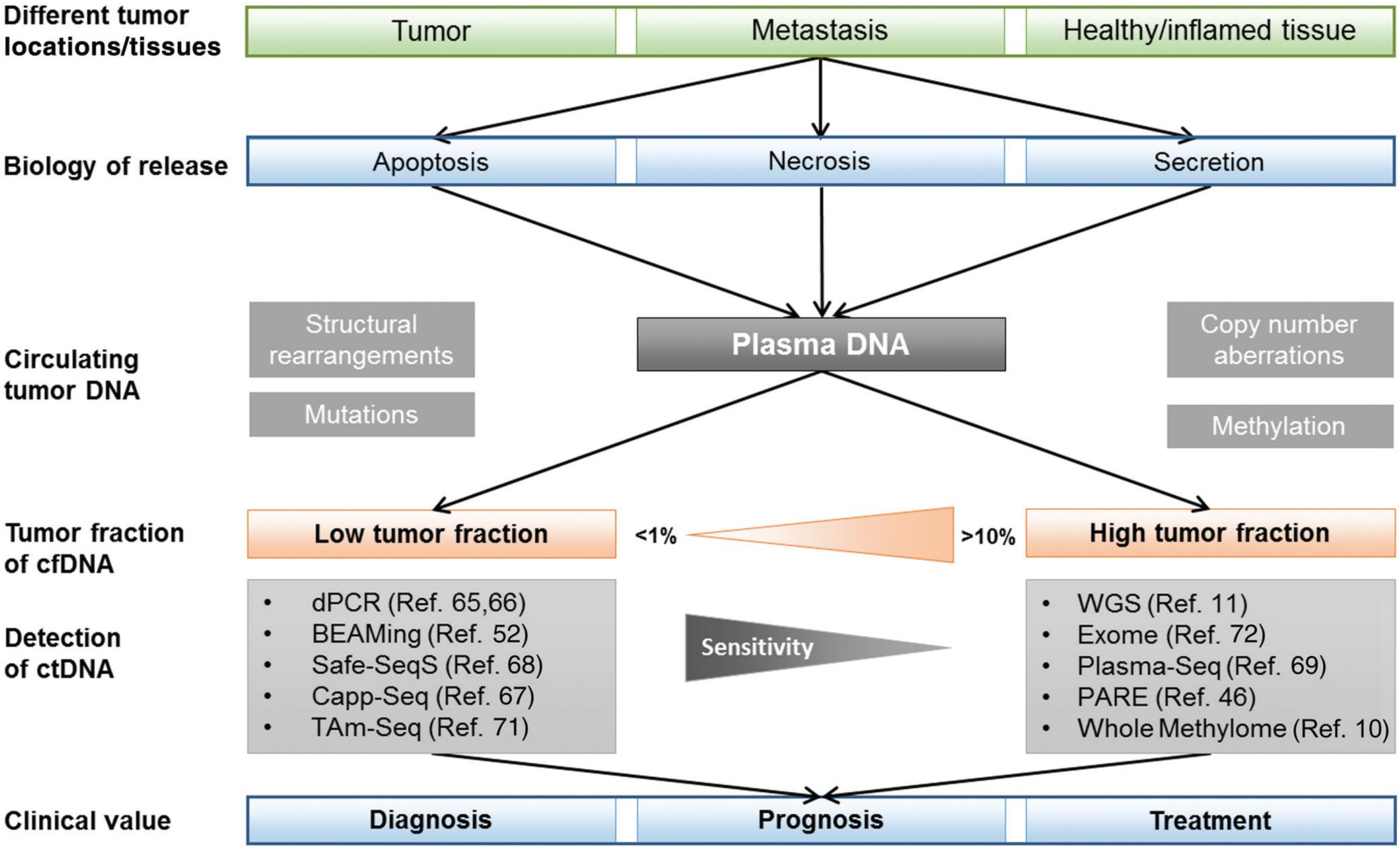




Re et al. Expert Rev Mol Diagn. 2014

Distribution of *TP53* mutations





Different tumor locations/tissues

Tumor | Metastasis | Healthy/inflamed tissue

Biology of release

Apoptosis | Necrosis | Secretion

Plasma DNA

Circulating tumor DNA

Structural rearrangements

Mutations

Copy number aberrations

Methylation

Tumor fraction of cfDNA

Low tumor fraction <1% >10% **High tumor fraction**

Detection of ctDNA

- dPCR (Ref. 65,66)
- BEAMing (Ref. 52)
- Safe-SeqS (Ref. 68)
- Capp-Seq (Ref. 67)
- TAm-Seq (Ref. 71)

Sensitivity

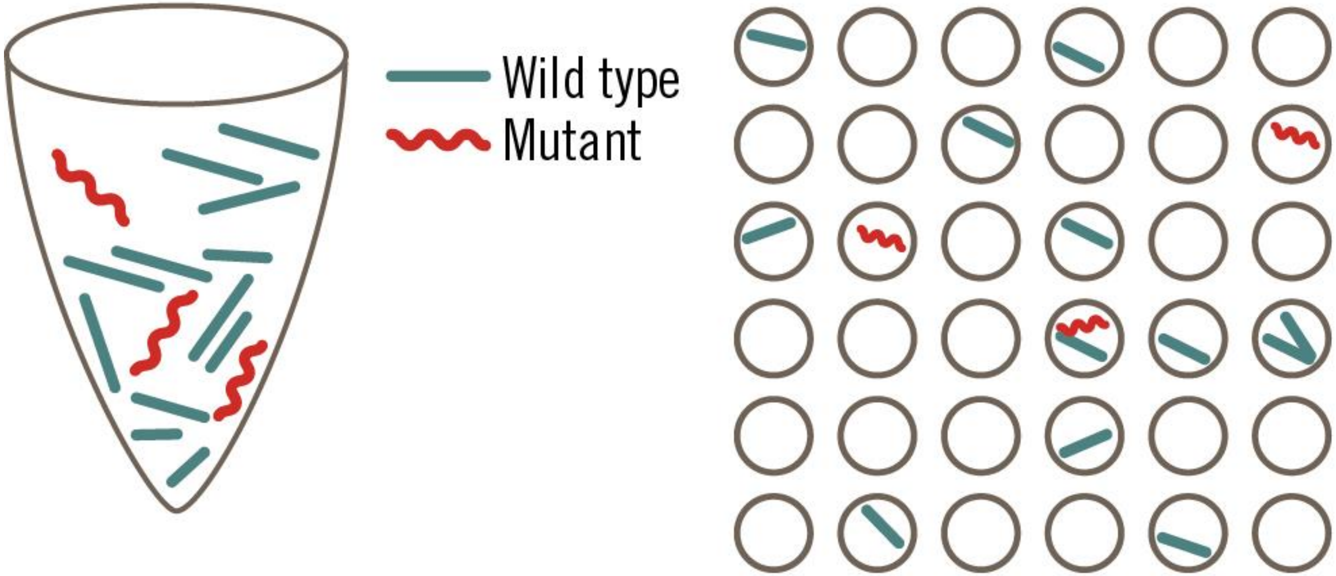
- WGS (Ref. 11)
- Exome (Ref. 72)
- Plasma-Seq (Ref. 69)
- PARE (Ref. 46)
- Whole Methylome (Ref. 10)

Clinical value

Diagnosis | **Prognosis** | **Treatment**

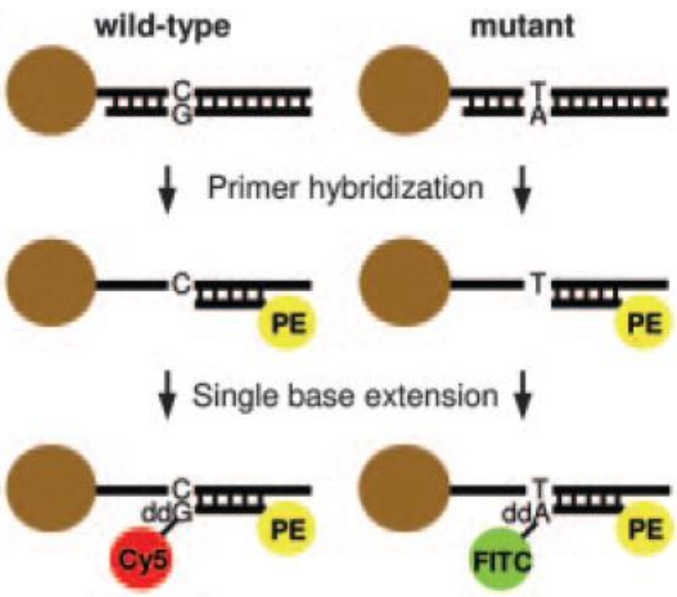
Heitzer et al. Clin Chem. 2015

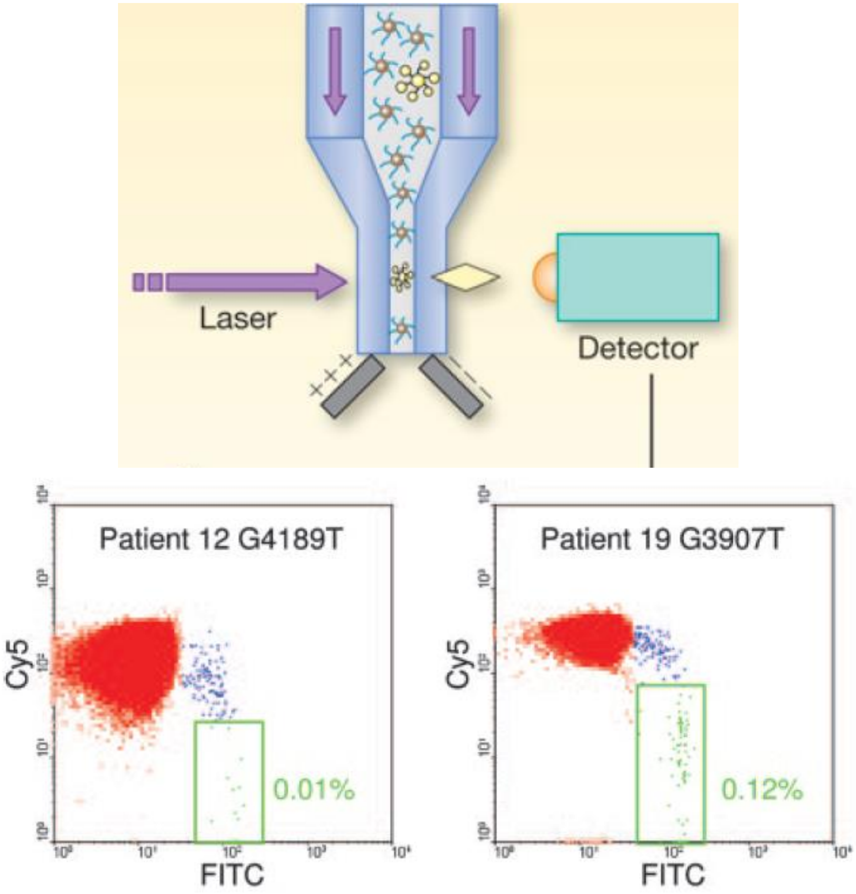
Digital PCR



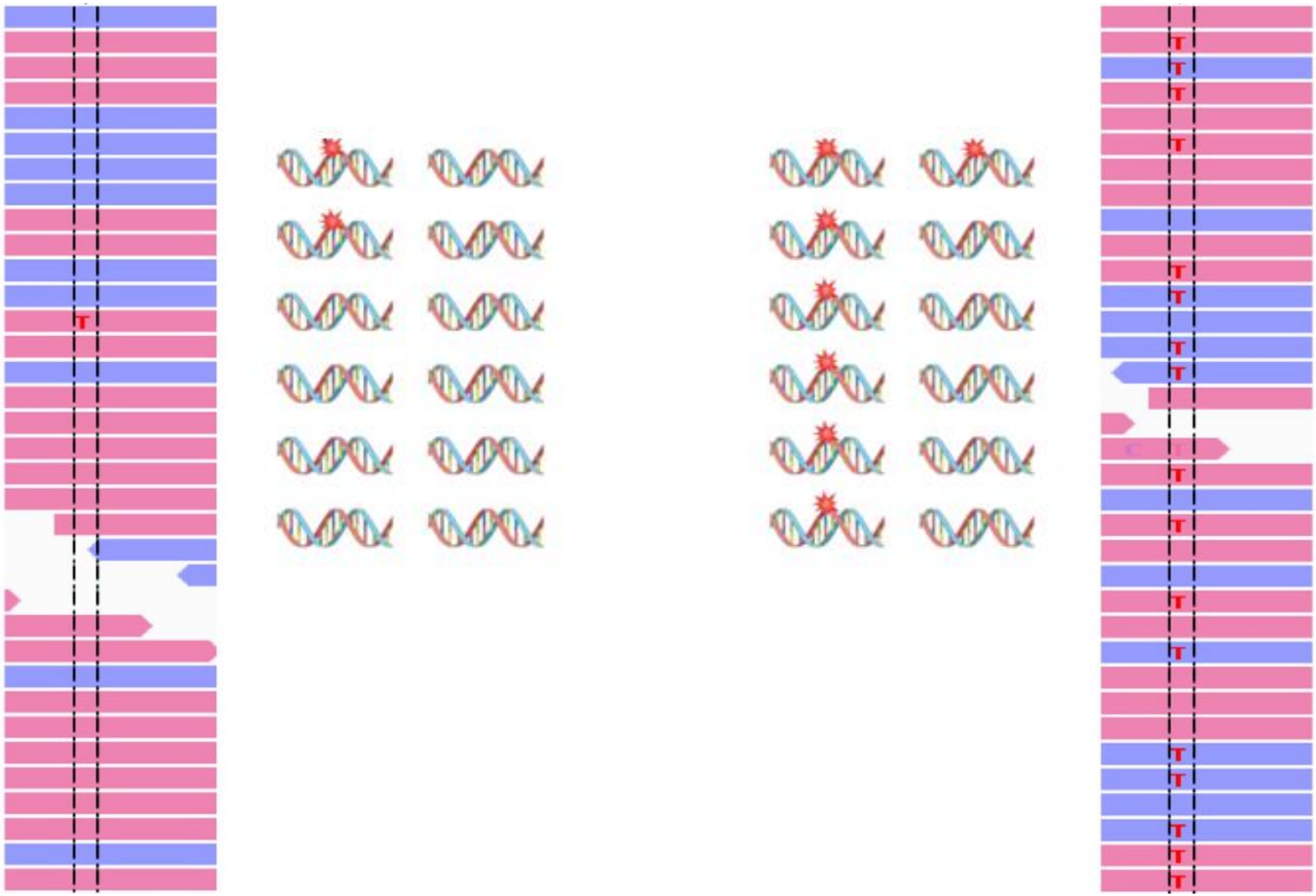
BEAMing method

(Beads, Emulsification, Amplification and Magnetics)



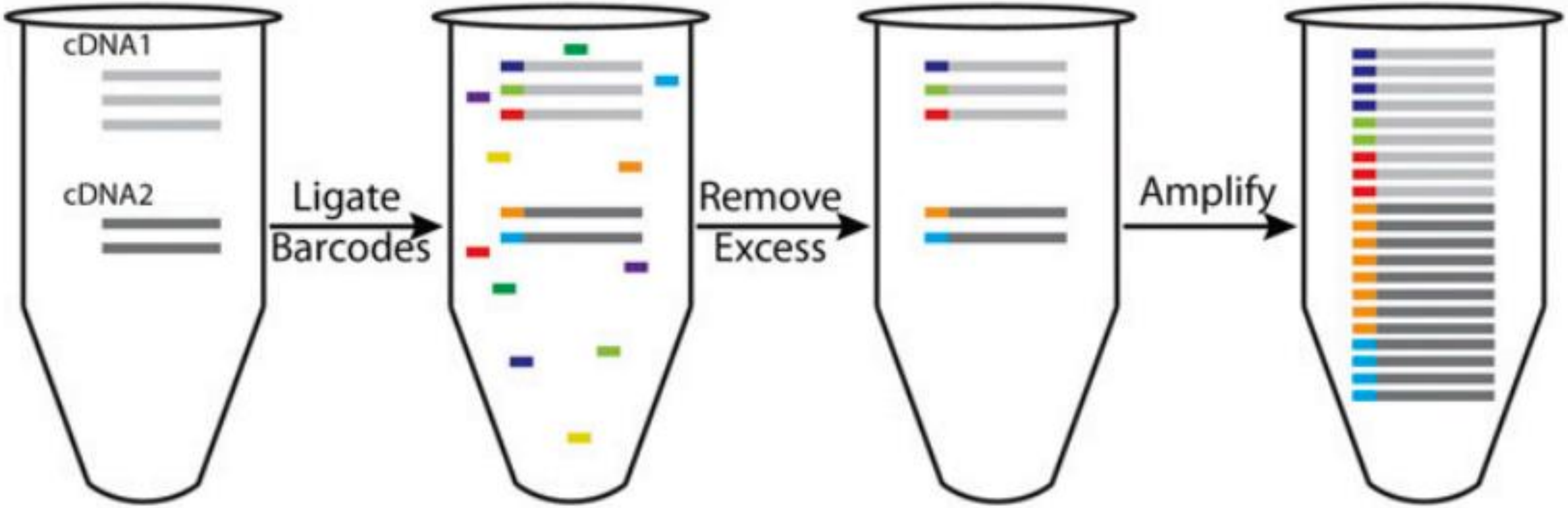


Deep sequencing by NGS

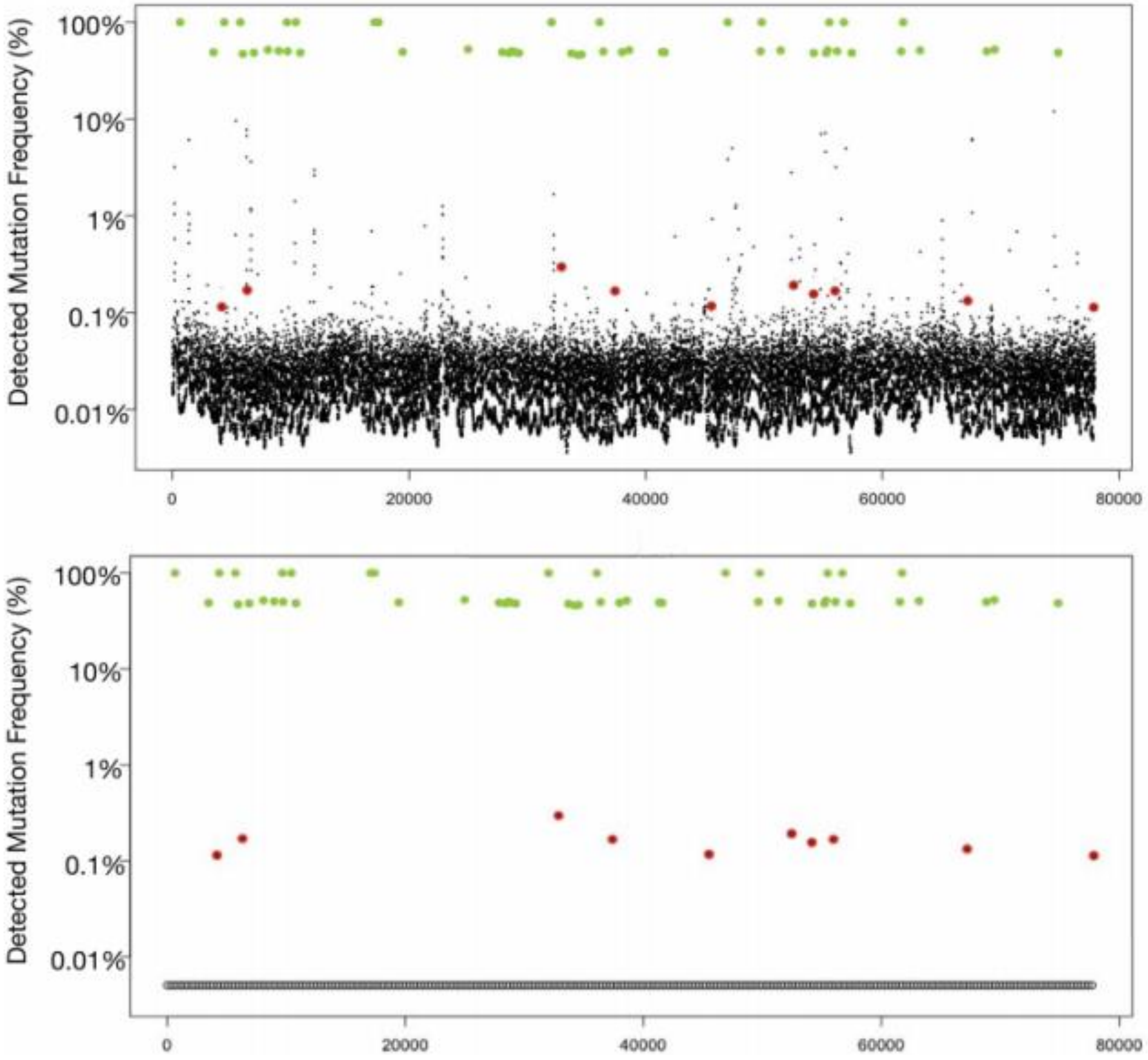


Digital sequencing

(Molecular barcoding)



Error reduction and increased sensitivity by digital sequencing



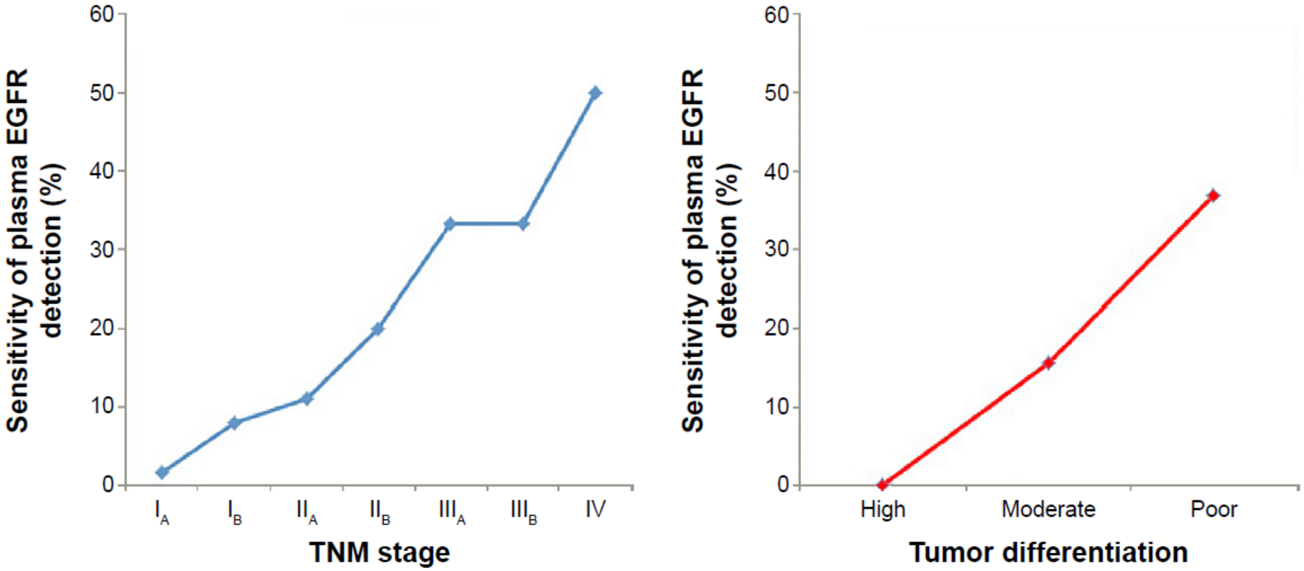
Lanman et al. PLOS One 2015

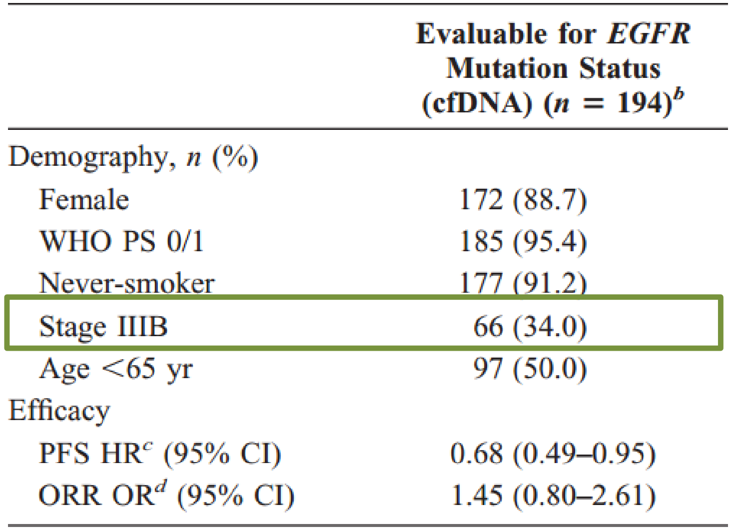
Gene mutations detected in ctDNA of NSCLC patients

Material	Method	Genes analyzed	Patients		Median progression free survival (PFS)		median overall survival (OS)		Medicine	Sensitivity (%)	Specificity (%)	References
			Number	Positive (%)	Mutation type (m)	Wild type (m)	Mutation type (m)	Wild type (m)				
Plasma	qPCR	KRAS	308	8.8	5.77	5.43	9.07	10.03	Cisplatin and docetaxel	NM	NM	[25]
Plasma	qPCR+ARMS	KRAS	246	17.5	3.0	5.6	4.8	9.5	Carboplatin and vinorelbine	90.0	NM	[32]
Plasma	PCR-RFLP	KRAS	273	12.8	2.5	8.8	16.9	20.3	EGFR-TKI	76.7	95.1	[31]
Plasma	Scorpion+ARMS	KRAS	49	4.08	1.4	5.4	2.5	18.8	Docetaxel plus erlotinib	100	92.3	[44]
Plasma	Scorpion+ARMS	KRAS	24	20.8	KRAS mutation status in ctDNA was not associated with PFS.		NM	NM	Pertuzumab plus erlotinib	100	100	[45]
Plasma	qPCR+NGS	KRAS	30	20.0	NM	NM	NM	NM	Radiation, chemotherapy, targeted systemic therapy and/or surgery	100	100	[36]
Peripheral blood	Colorimetric membrane array	KARS	209	28.2	NM	NM	NM	NM	NM	83.1	96.4	[46]
Peripheral blood	Weighted chemiluminescent membrane array	KARS	209	31.6	NM	NM	NM	NM	NM	93.0	94.2	[46]
Plasma	qPCR	EGFR	56	23.2	11.2	2.7	21.8	5.8	Gefitinib	NM	NM	[26]
Plasma	Digital PCR	EGFR	35	43.0	NM	NM	NM	NM	EGFR TKIs	92	100	[35]
Serum	PCR+direct sequencing	EGFR	27	37.0	NM	NM	NM	NM	Gefitinib	75.0	71.4	[30]
Serum	nested PCR+ sequencing	EGFR	44	9.1	NM	NM	41.9	17.9	EGFR TKIs	NM	NM	[47]
Plasma	Scorpion+ARMS	EGFR	54	47	NM	NM	NM	NM	Gefitinib/erlotinib	70	85	[48]
Serum		EGFR	42	16.7	174 days	58 days	387 days	228 days	Gefitinib	75	97.1	[49]
Serum		EGFR	27	48.1	200 days	46 days	611 days	232 days	Gefitinib	50.0	85.7	[50]
Plasma		EGFR	49	20.4	Exon 19/21: 18.3 T790M: 3.9	4.0	Exon 19/21: 39.6 T790M: 24.2	17.8	Docetaxel plus erlotinib	66.7	100	[44]
Serum		EGFR	194	23.7	PFS was significantly longer with gefitinib than carboplatin/ paclitaxel in patients with EGFR mutation in ctDNA		EGFR mutation status in ctDNA was not associated with longer OS.		Gefitinib or carboplatin plus paclitaxel	43	100	[51]
Plasma		EGFR	24	16.7	EGFR mutation status in ctDNA was associated with longer PFS		NM	NM	Pertuzumab plus erlotinib	100	100	[45]
Plasma	DHPLC	EGFR	230	34.3	11.1 (8.7–16.8)	5.9 (2.1–9.7)	EGFR mutation status in ctDNA was not associated with longer OS		Gefitinib	81.8	89.5	[34]
Serum	Cycleave RT-PCR	EGFR	24	25.0	EGFR mutation status in ctDNA was not associated with longer PFS		EGFR mutation status in ctDNA was not associated with longer OS		NM	NM	NM	[52]
Plasma	Wild-inhibiting PCR Mutation-biased PCR	EGFR	39	Exon 19,: 44.7 L858R, 8.7	NM	NM	NM	NM	Operation or chemotherapy	NM	NM	[53]
Plasma	ME-PCR	EGFR	627	Exon19, 6.0	NM	NM	NM	NM	EGFR-TKI	Exon19, 100	Exon19, 50	[28]

Mutation detection rate according to stages

(Scorpion-ARMS method)





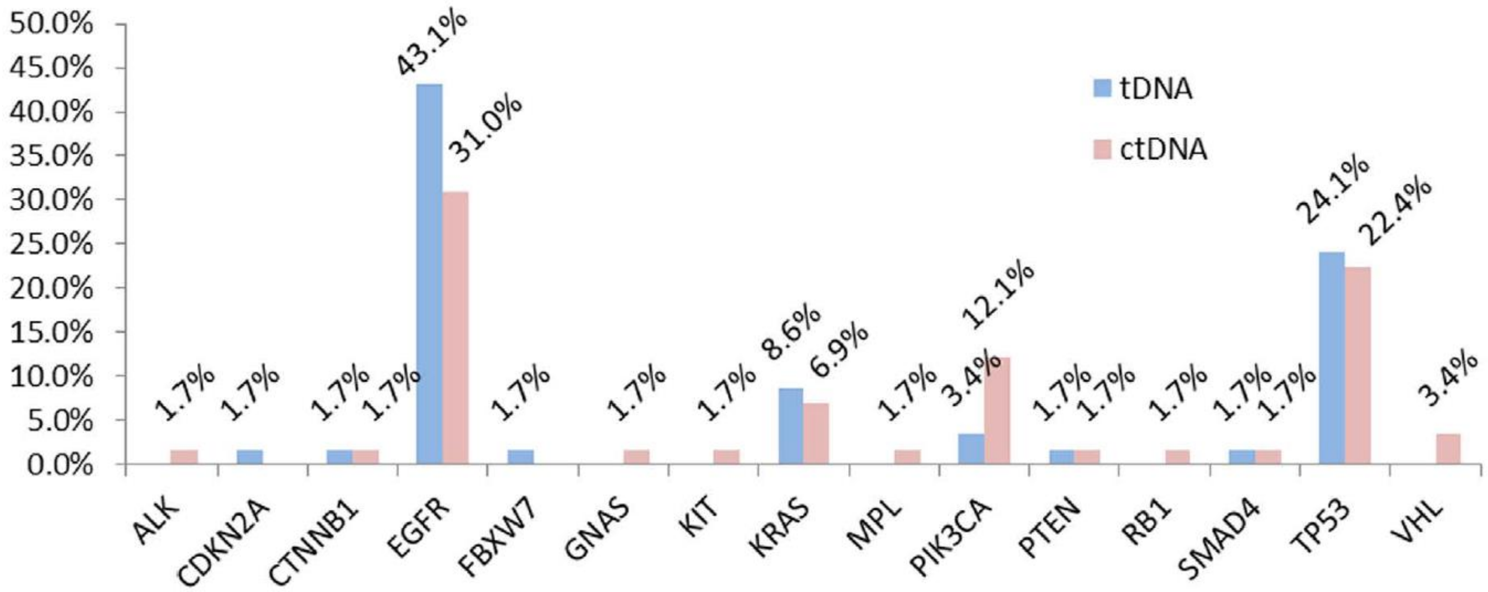
	Evaluable for *EGFR* Mutation Status (cfDNA) (*n* = 194)[b]
Demography, *n* (%)	
Female	172 (88.7)
WHO PS 0/1	185 (95.4)
Never-smoker	177 (91.2)
Stage IIIB	66 (34.0)
Age <65 yr	97 (50.0)
Efficacy	
PFS HR[c] (95% CI)	0.68 (0.49–0.95)
ORR OR[d] (95% CI)	1.45 (0.80–2.61)

Goto et al. J Thorac Oncol 2012

Guo et al. OncoTargets and Therapy 2015

Mutation detection in early-stage NSCLC

(Next-generation sequencing)

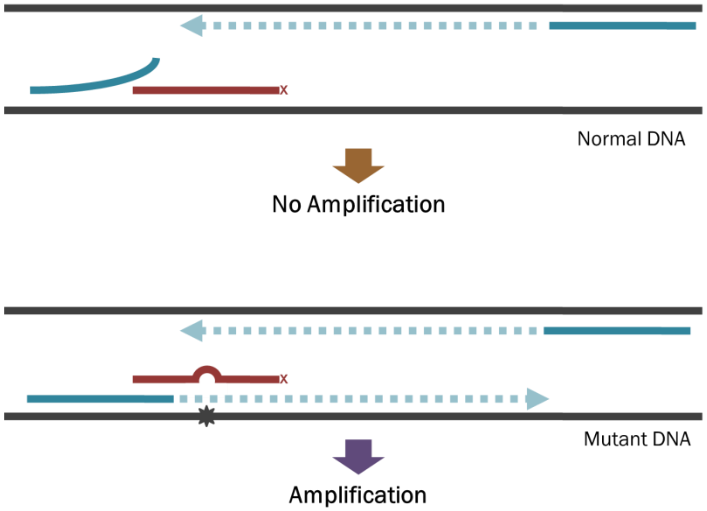


- 58 Early-stage NSCLC (Ia, Ib, and IIa)
- 45 (77.6%) contained mutations in more than one genes
- Comparison with tumor: sensitivity 53.8% / specificity 47.3%

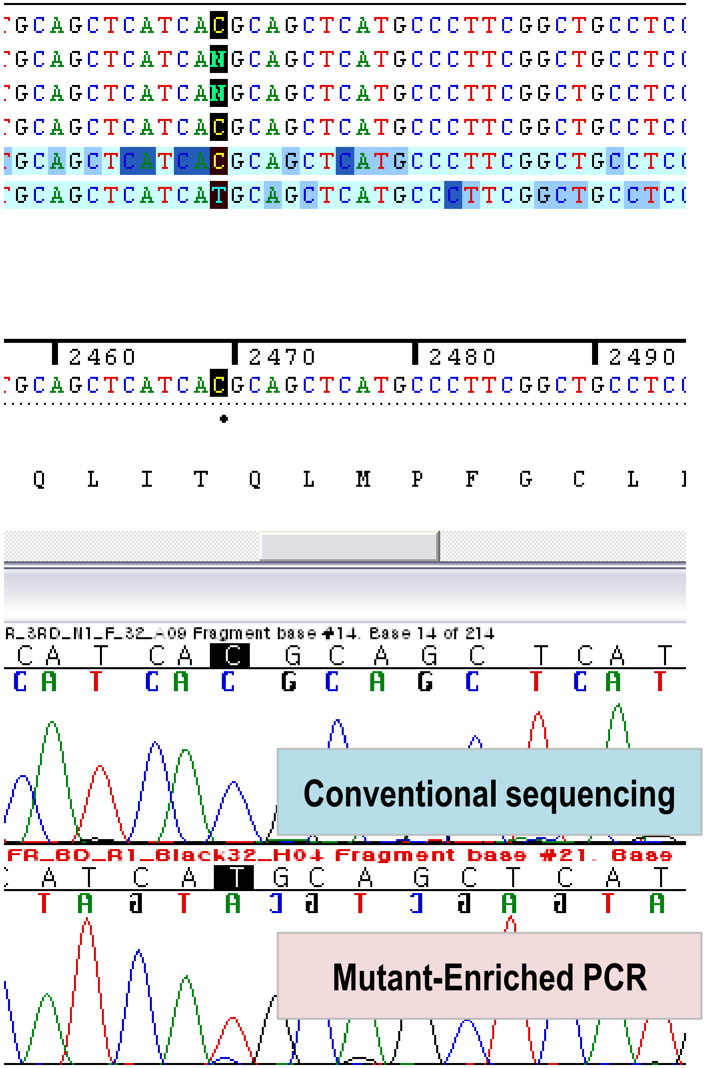
Luo et al. Sci Reports. 2014

MEMO-PCR

(*EGFR* T790M mutation in 40 lung cancer)



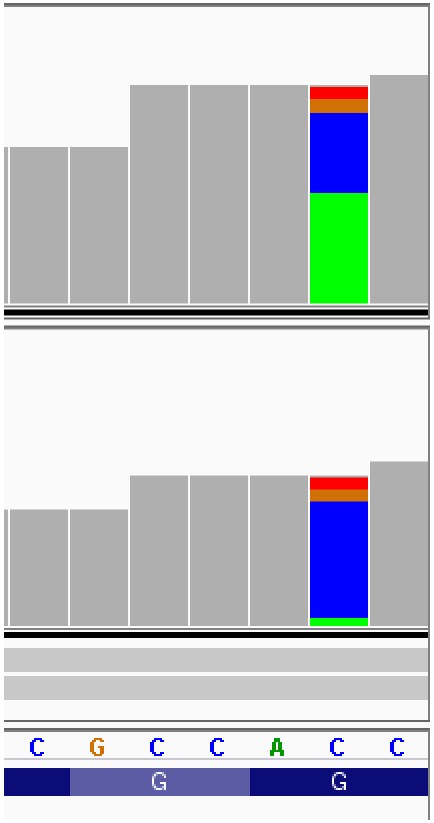
		After Chemotherapy	
		Positive	Negative
Initial	Positive	9	11
	Negative	3	17



MEMO-PCR & NGS using plasma

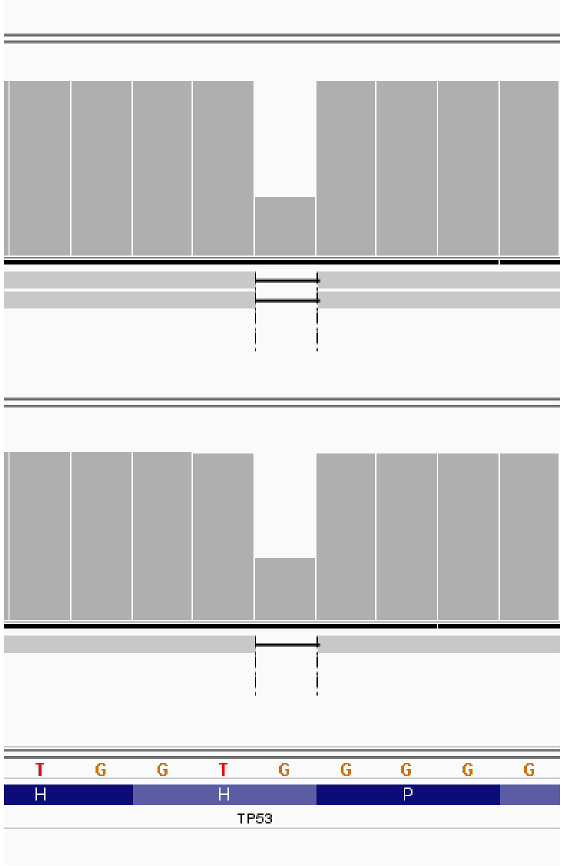
MEMO-PCR
& deep sequencing

NGS deep
sequencing



KRAS p.G13D

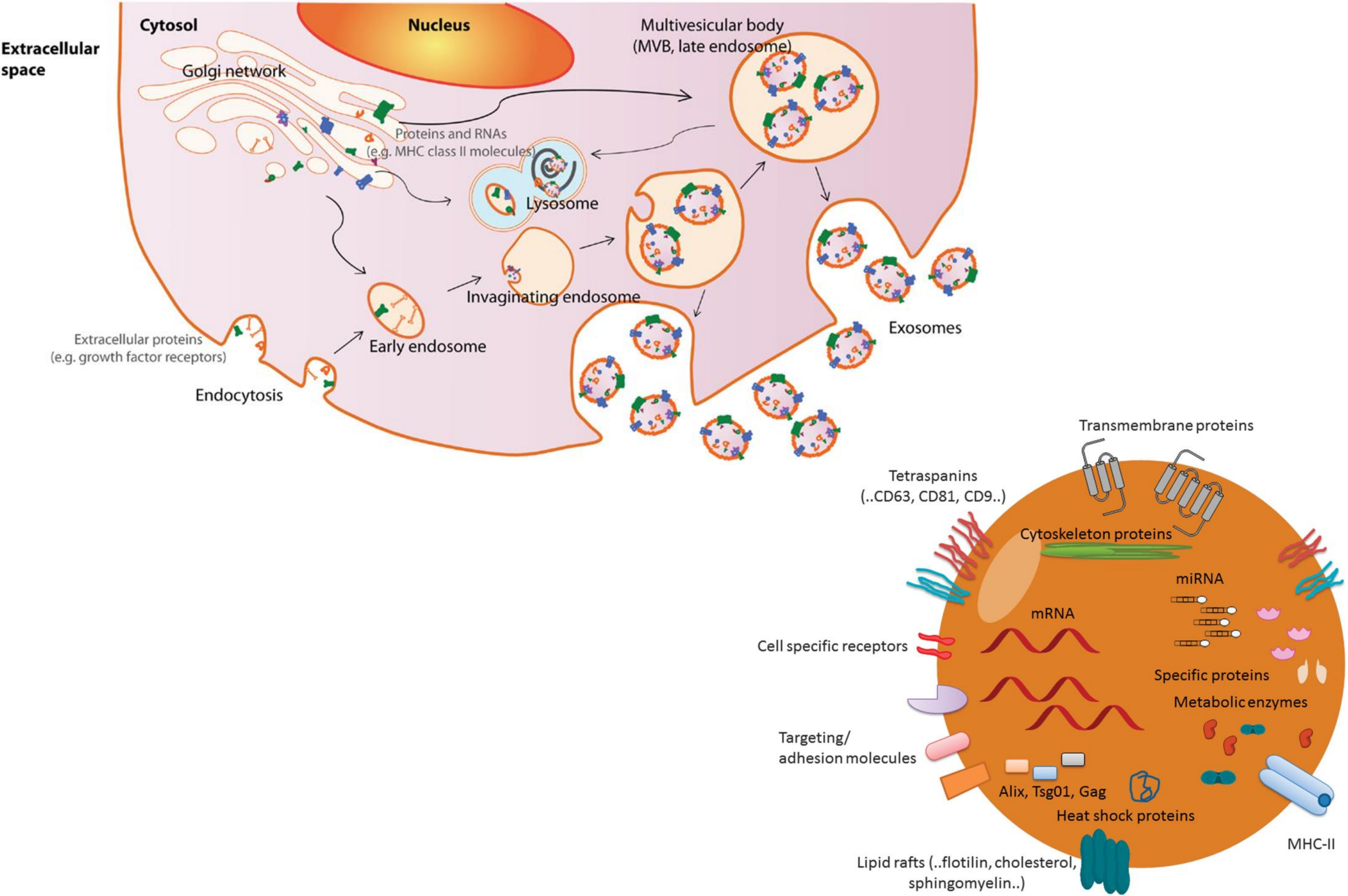
Lung adenocarcinoma
(Stage IIb)



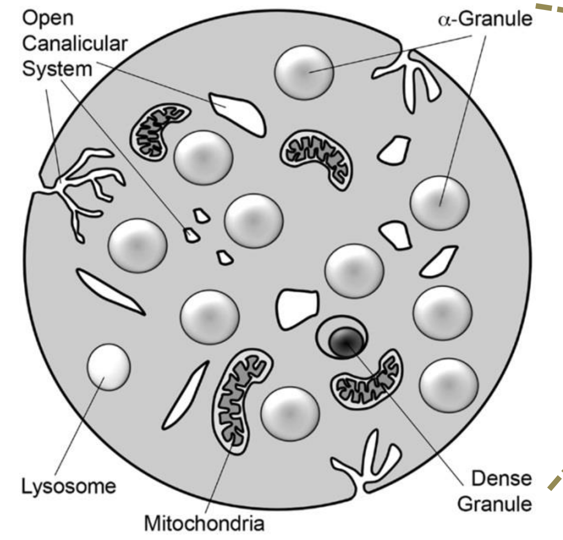
TP53 c.532delC

Colon adenocarcinoma
(Stage IV)

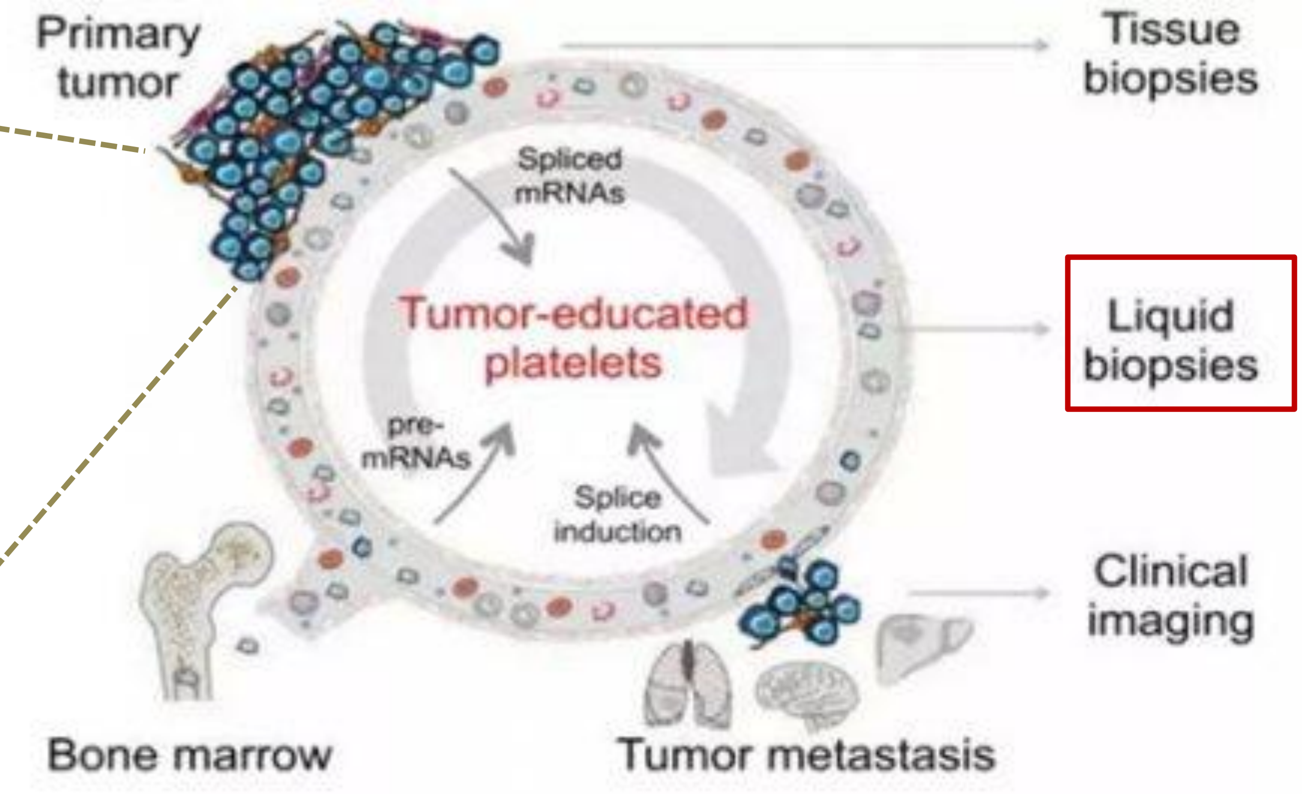
Exosomes, ctRNA, miRNA

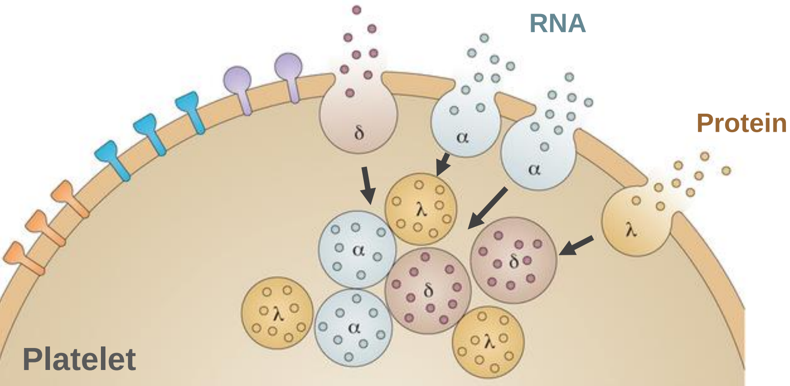


Tumor-educated platelets

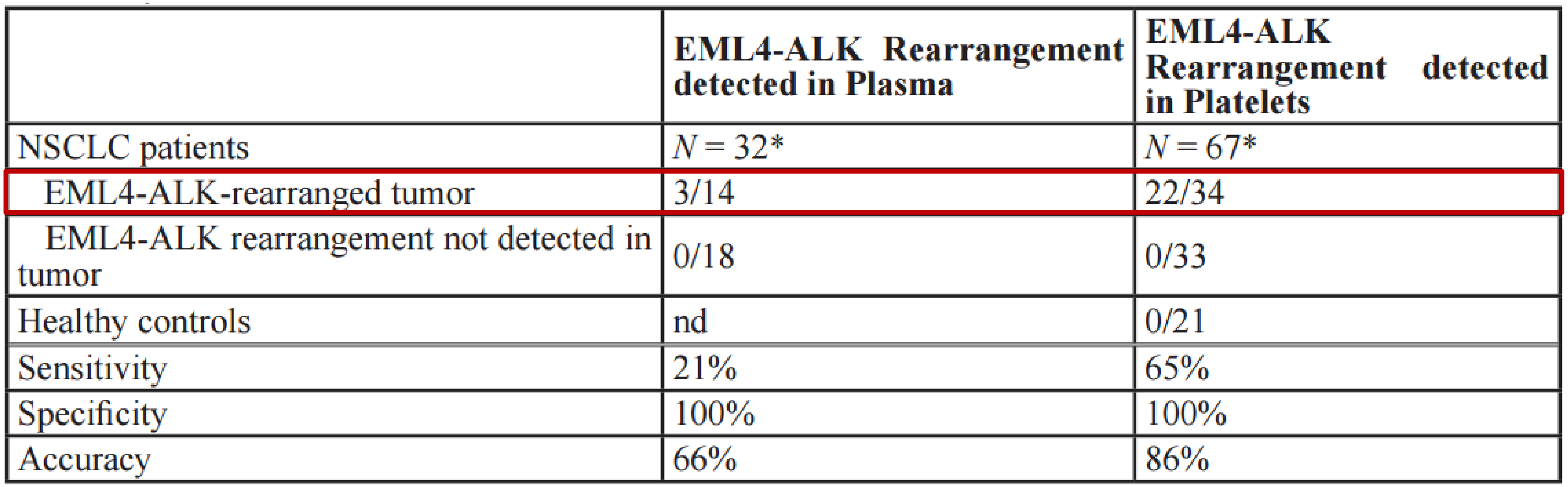


Platelet

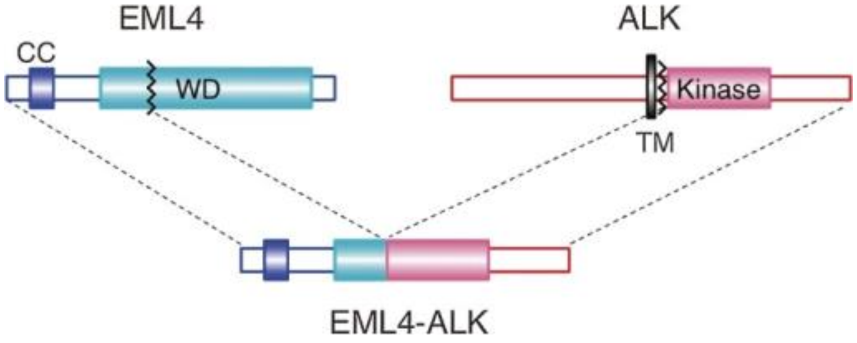


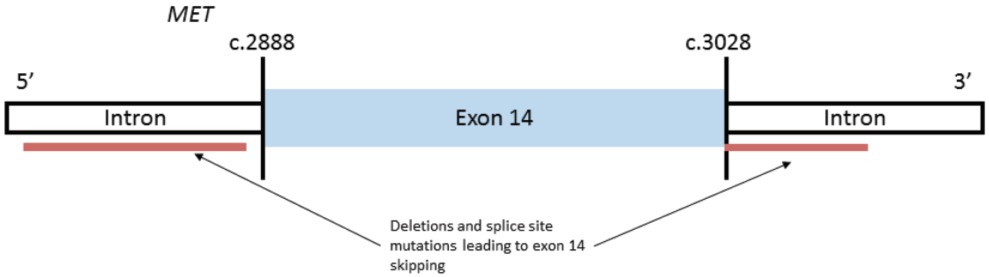


Detecting RNA fusion in tumor-educated platelets



	EML4-ALK Rearrangement detected in Plasma	**EML4-ALK Rearrangement detected in Platelets**
NSCLC patients	*N* = 32*	*N* = 67*
EML4-ALK-rearranged tumor	3/14	22/34
EML4-ALK rearrangement not detected in tumor	0/18	0/33
Healthy controls	nd	0/21
Sensitivity	21%	65%
Specificity	100%	100%
Accuracy	66%	86%





Nilsson et al. Oncotarget. 2016

Summary

- Blood-based tumor diagnostics are evolving rapidly and recent studies show promising results.
- Blood has become an attractive specimen for early detection of lung cancers.
- Technical optimization, further validation, standardization and practical application may be increase the utility of blood-based methods.