Single cell genetic analysis helps validating cytopathological identification of CTCs in patients with Clear Cells Renal Carcinoma

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PARIS DESCARTES

ASSISTANCE PUBLIQUE HÔPITAUX DE PARIS NECKER-ENFANTS MALADES

Rarecells

Disclosure

Inventor/coinventor of ISET patents
Founder and CSO of Rarecells

I do not receive conpensation from Rarecells
nor from any other company

I have a triple task:
Teaching (University Paris Descartes)
Developing and implementing new tests (Hôpital Necker)
Research activity (INSERM Unit)

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Overview

Purpose

Technical note on ISET

Published results

New workflows

Molecular analyses of CRC

Molecular analysis of CTC from Kidney cancer

Toward a diagnostic use of CTC in predictive oncology

CS-CTC= EpCam and CK positive, CD45 negative

FDA clearence: clinical validity but clinical utility not demonstrated or demonstrated in M+ patients

Sensitivity and specificity problems

Reproducible data does not mean clinically useful data

Underevaluation of CTC heterogeneity

Confounding results for molecular analyses and theranostic application

Diagnosis of CTC: diagnosis of tumor and clinical utility for patients

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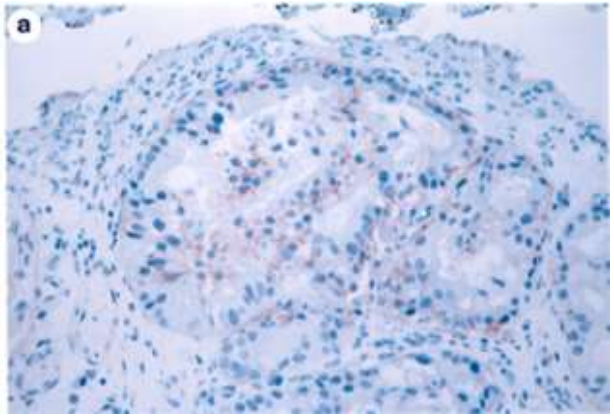
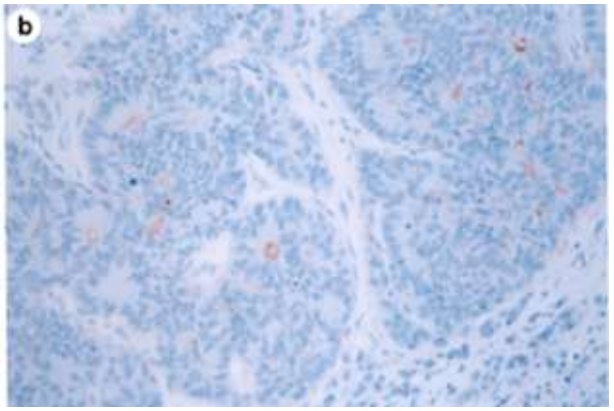
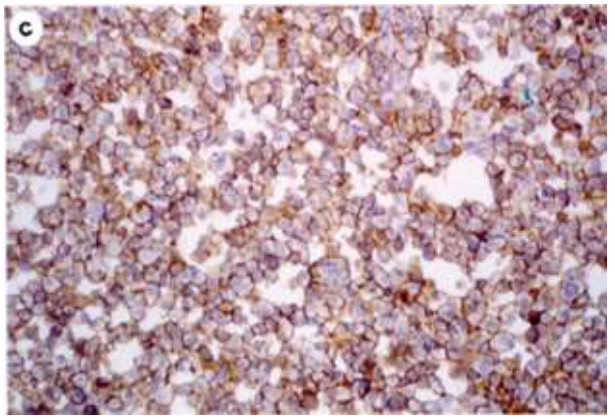
Tumor cells with epithelial markers in tumors: minority



34 PAC
20 (59%) tumors with pos. cells
5-70% range of pos cells



27 HCC
5 (18%) tumors with pos. cells
1-50% range of pos. cells



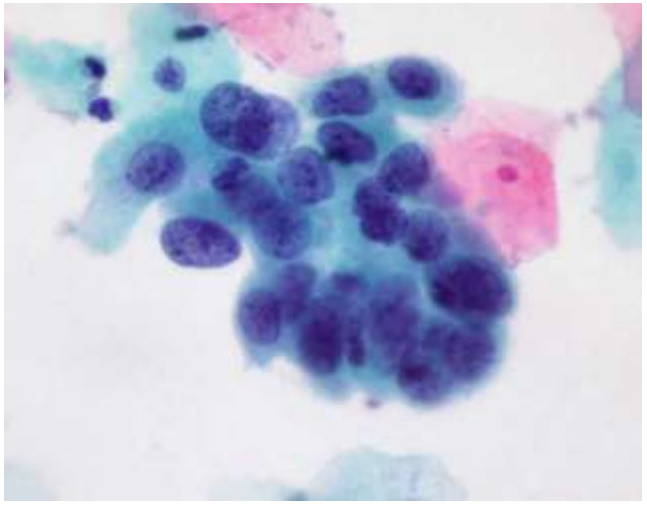
HCC and PAC
derived cell lines
95-99% pos. Cells

A. Sabile et al Am. J. Clin. Pathol. 112: 171-178, 1999

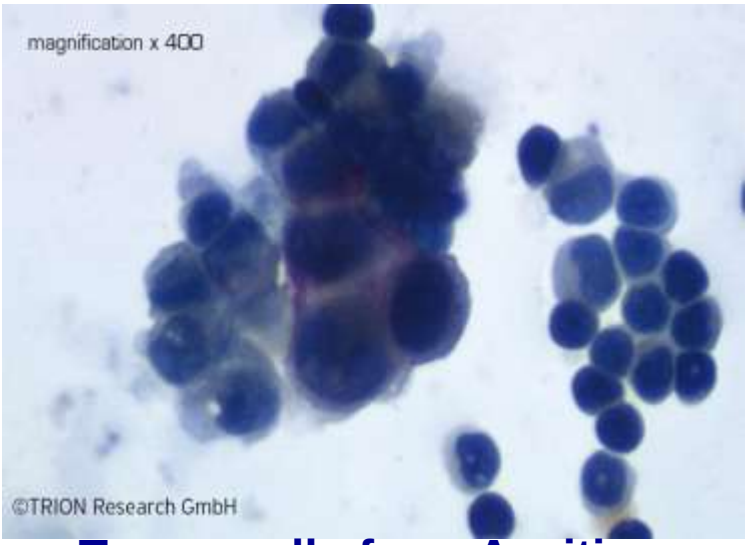
Epithelial to Mesenchymal transition + tumor heterogeneity

Why Epithelial markers are used to isolate CTC?

Diagnosis of CTC in Oncology

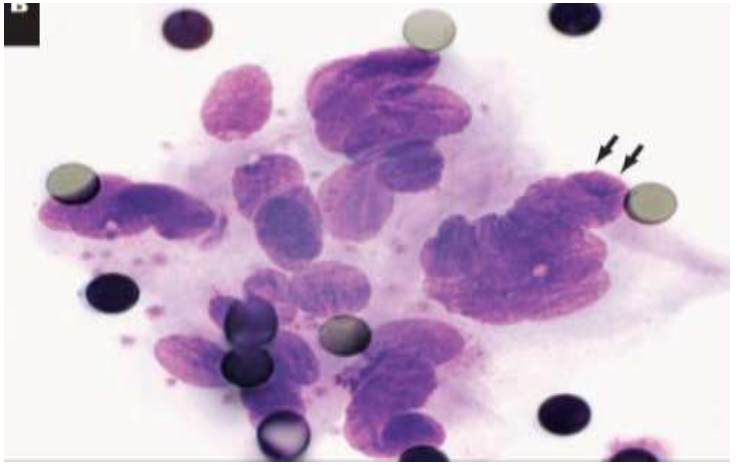


Tumor cells from Cervix



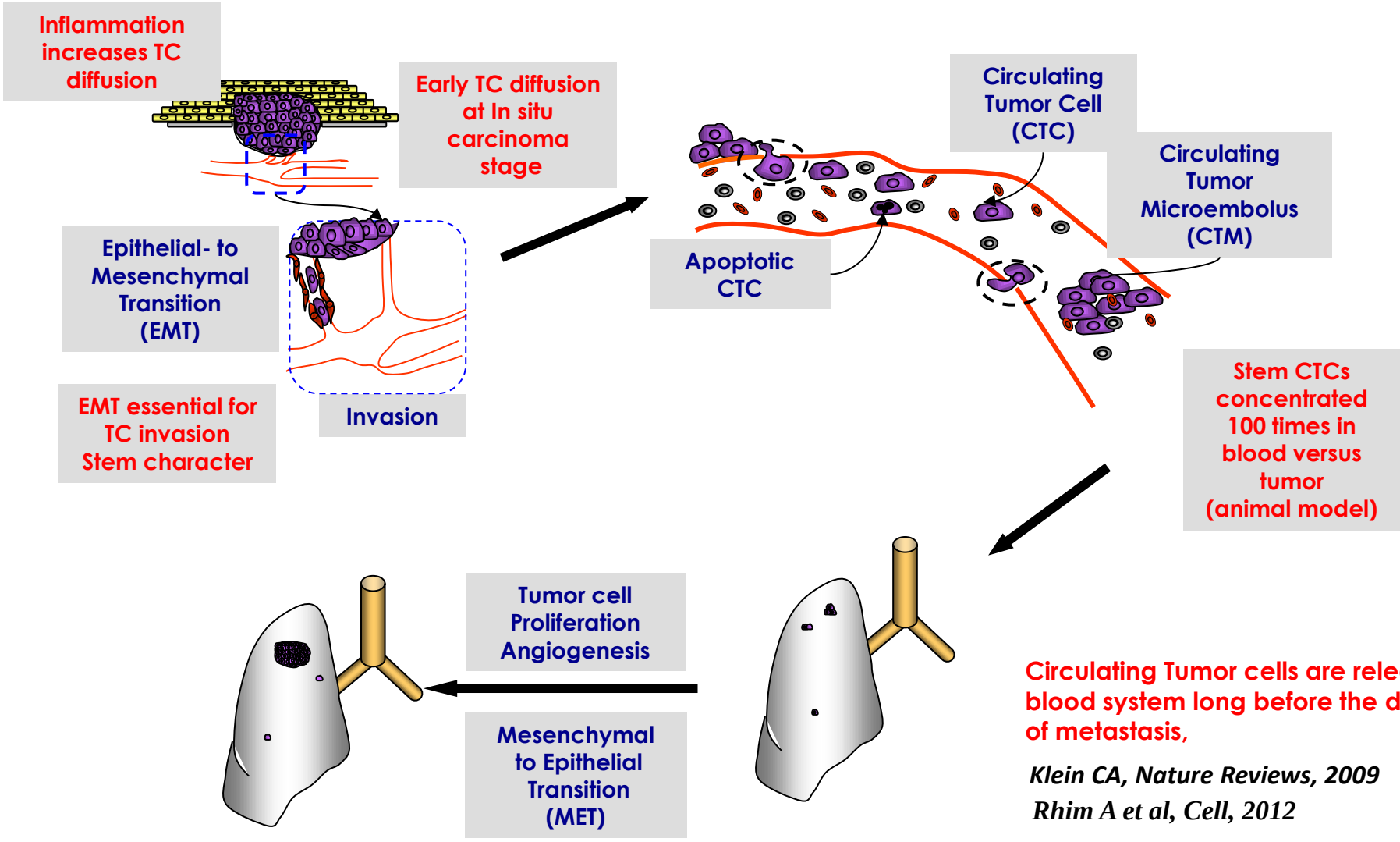
Tumor cells from Ascitis

Tumor cells from Urine



Tumor cells from Blood

CTC- Circulating Tumor Cells



Circulating Tumor cells are released into the blood system long before the development of metastasis,

Klein CA, Nature Reviews, 2009

Rhim A et al, Cell, 2012

Tumor cells heterogeneity in tumor tissue

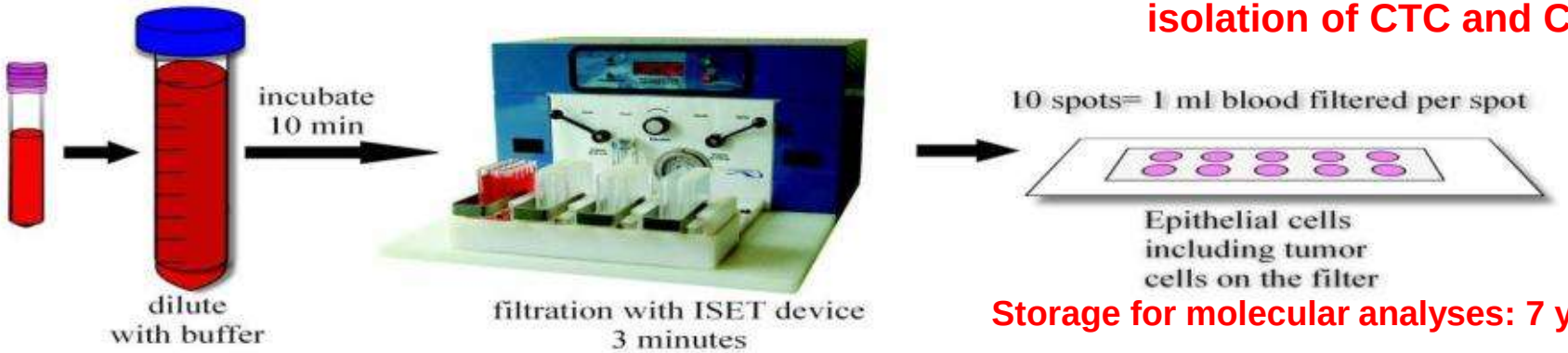
Gerlinger et al, NEJM, 2012

Paterlini Brechot , Cancer letters 2007, updated

ISET Isolation by Size of Tumor/Trophoblastic cells

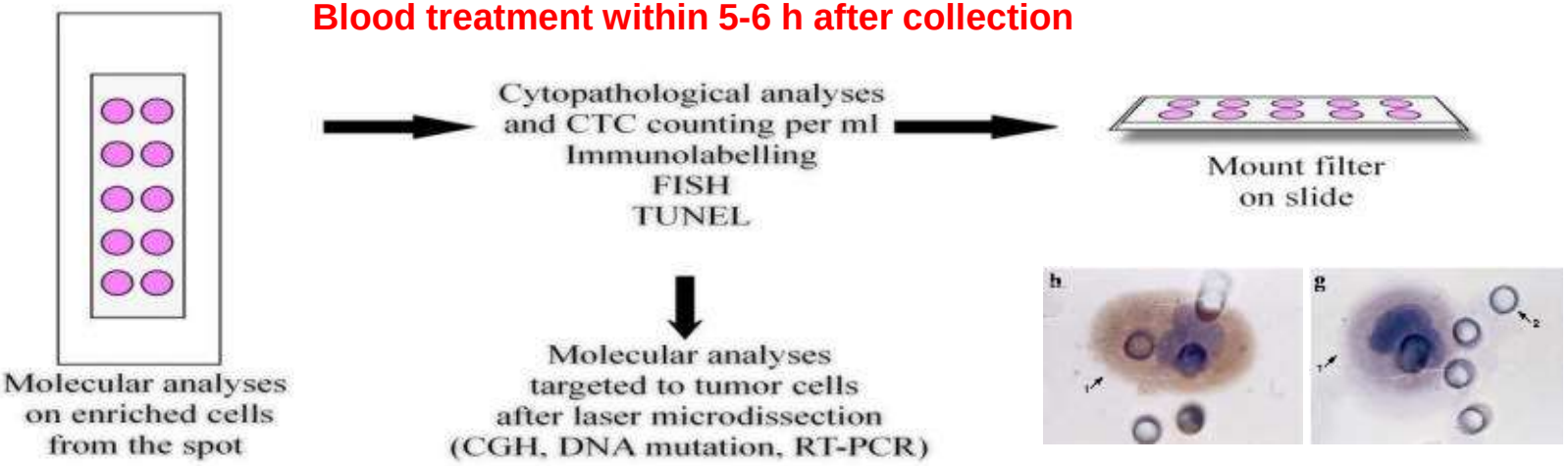
Leukocytes are the smallest cells in the body

Patented combination of parameters allowing very sensitive and rapid isolation of CTC and CFTC



Storage for molecular analyses: 7 years

Blood treatment within 5-6 h after collection



Open system allowing all types of analysis on CTCs

Vona et al, Am J Pathol, 2000

ISET is diagnostic and more sensitive than CellSearch

independent studies *in vivo*: % of positive patients

ISET (size+ cytopathology) versus

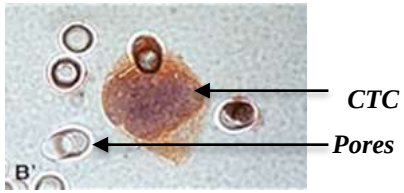
Type of cancer	Number of patients	Reference	ISET % of patients with CTC	CellSearch % of patients with CEpC (cut-off)
Non-Small Cell Lung cancer	210 (non-metastatic and metastatic)	***Hofman V et al, Int J of Cancer 2010***	50%	39% (≥ 1) 21% (≥ 2)
	20 (metastatic)	***Farace F et al, Br J of Cancer, 2011***	100%	45% (≥ 1) 25% (≥ 2) 15% (≥ 5)
	40 (IIIA - IV)	***Krebs M et al, J. Thoracic Onc. 2011***	80%	23% (≥ 2)
Prostate cancer	20 (metastatic)	***Farace F et al, Br J of Cancer, 2011***	100%	90 % (≥ 1) 60 % (≥ 5)
Breast cancer	20 (metastatic)	***Farace F et al, Br J of Cancer, 2011***	85%	75% (≥ 1) 40% (≥ 5)
Pancreatic cancer	54 (non-metastatic and metastatic)	***Khoja L et al, Br J of Cancer, 2011***	93%	40% (≥ 1)

ISET detects CTCs in higher numbers and in more patients

CTM (Circulating Tumor Microemboli) are detected by ISET only

ISET - RARECELLS

ISET Enrichment/detection principle: *Size, cytopathology*



Cytopathological analysis is diagnostic in Oncology

ISET allows the diagnosis of CTC

Work of process: *Rapid, easy to use, efficient*

ISET isolates all types of CTC (all types of cancer) and all types of CRC

Scientific results: *Sensitivity, Diagnostic specificity*

- ***ISET is diagnostic for CTC***
- ***Unparalleled sensitivity (1/10 ml)***
- ***Identifies the most malignant CTCs (expressing Vimentin)***
- ***Identifies Circulating Tumor Microemboli (CTM)***
- ***ISET allows molecular analysis of CTCs***

Low cost, filters and cells storable; multiplex tests

Aspiration controlled by a pump allows to optimize filtration of all types of blood samples

CYTOPATHOLOGICAL DEFINITION OF CTC

- Nuclear size equal or larger than 16 microns,
- Irregularity of the nulear countour
- Irregularity of the chromatin
- Presence of a visible cytoplasm
- High nuclear to cytoplasmic ratio (>0.8)

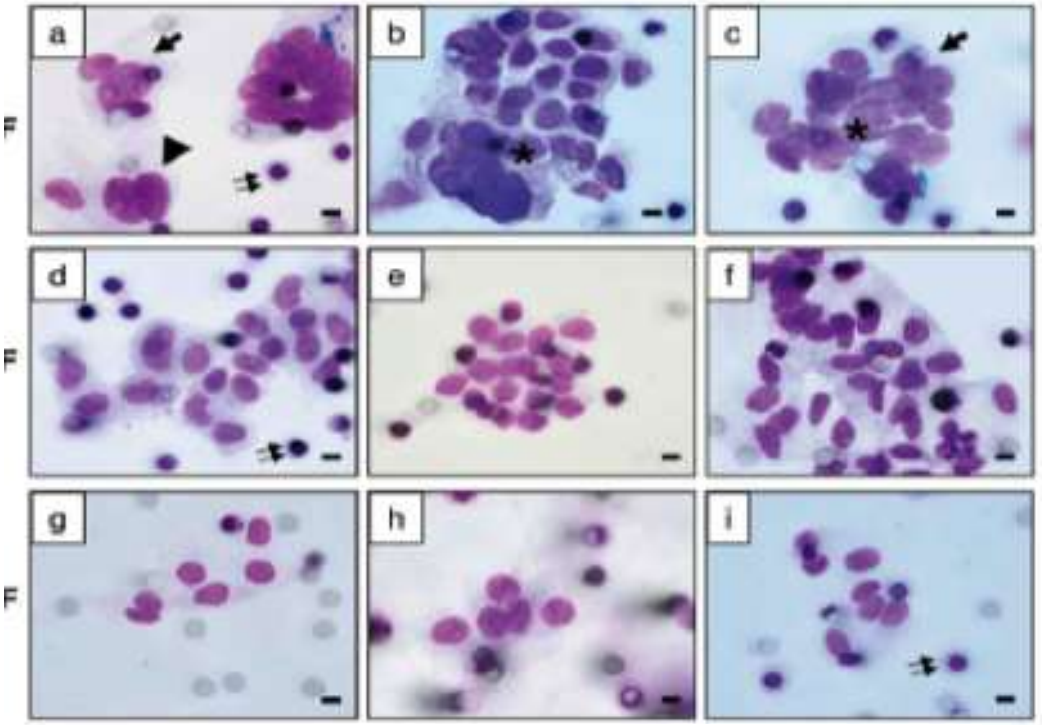
SPECIFICITY

Heterogeneity of circulating rare cells

Tumor

Atypical

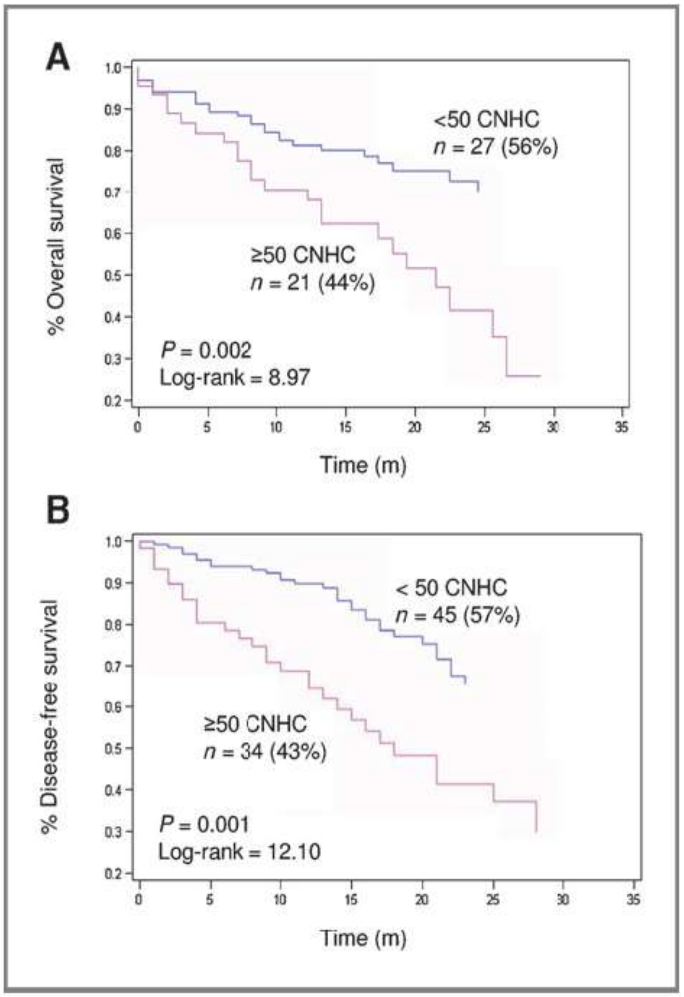
Normal

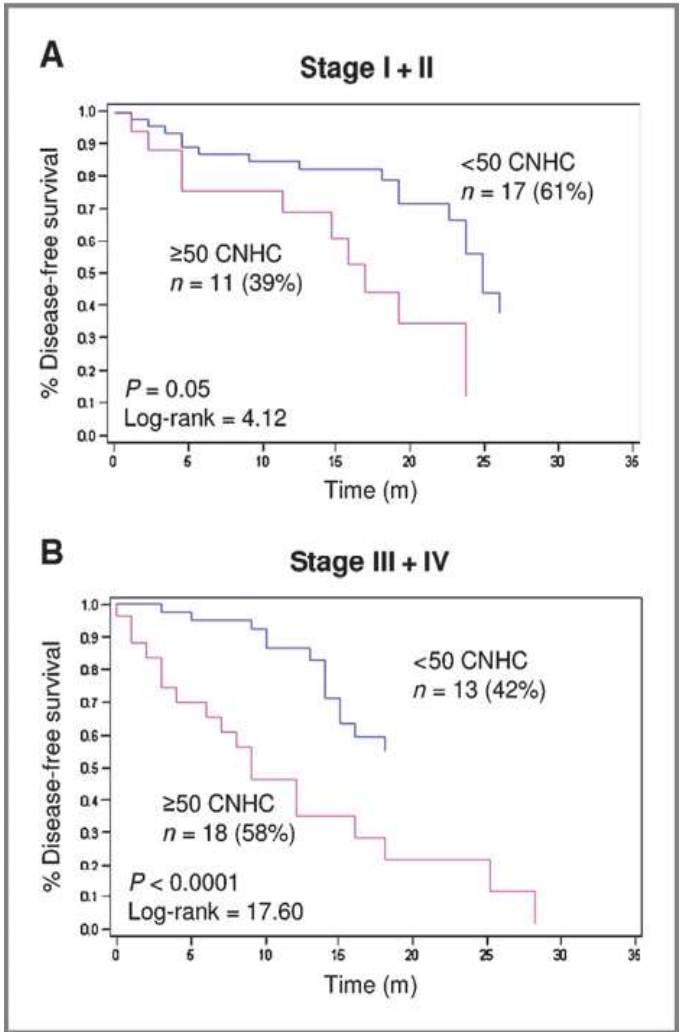


Hofman et al, Clinical Cancer Research, 2011

Preoperative CTC detection using the ISET method for patients with lung cancer is a new prognostic biomarker

208 patients **49% CNHC** **36% CTC**



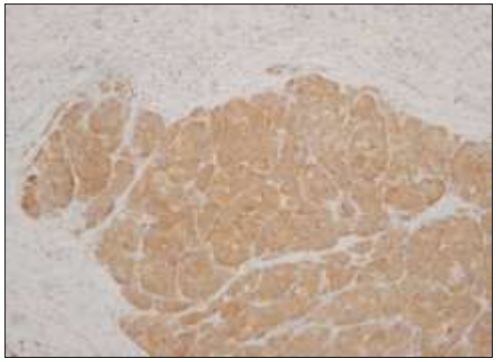


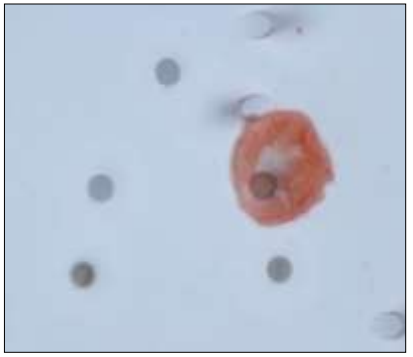
Hofman Clinical Cancer Research 2011

Diagnostic value of ICC for the detection of BRAF^{V600E} mutation on CTC isolated by ISET from metastatic melanoma patients

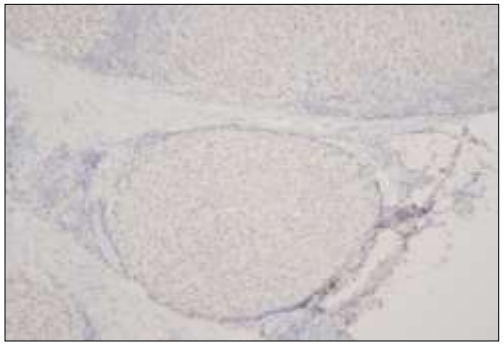
Hofman V et al , J Invest Dermatology, 2012

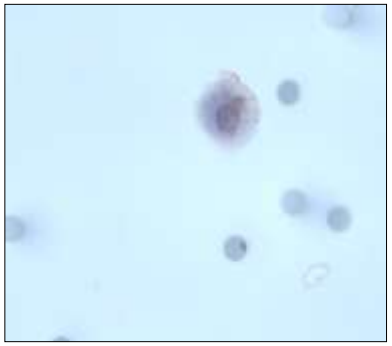
Patient 1



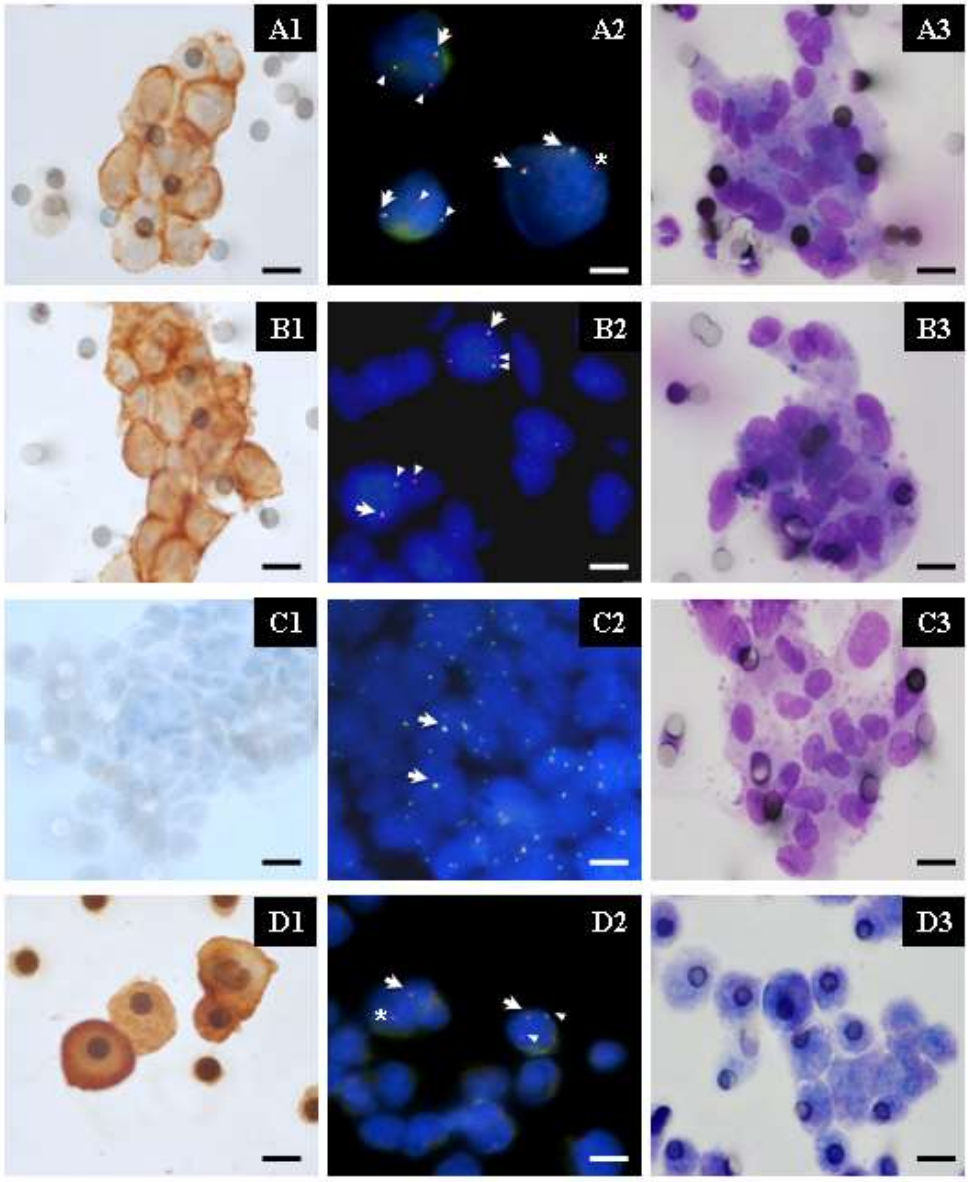


Patient 2





- BRAF^{V600E} mutation searched in 98 tumour tissues and CTCs by pyrosequencing (T) and by ICC using the VE1 antibody (T, CTC).
- 87/98 (89%) patients with CTCs
- 53/98 (54%) patients with BRAF^{V600E} mutation detected by pyrosequencing in T
- Consistent results (T, CTC)
- 8/87 (8%) patients had CTCs positive by ICC but no mutation detected on tumour tissues.
- The ICC detection of BRAF^{V600E} mutation on CTCs isolated by ISET is a very sensitive and specific method



EML4-ALK-gene rearrangement is consistently found in CTC/CTM isolated by ISET and in the tumorous tissue

Ilie M et al, Annals of Oncology, 2012

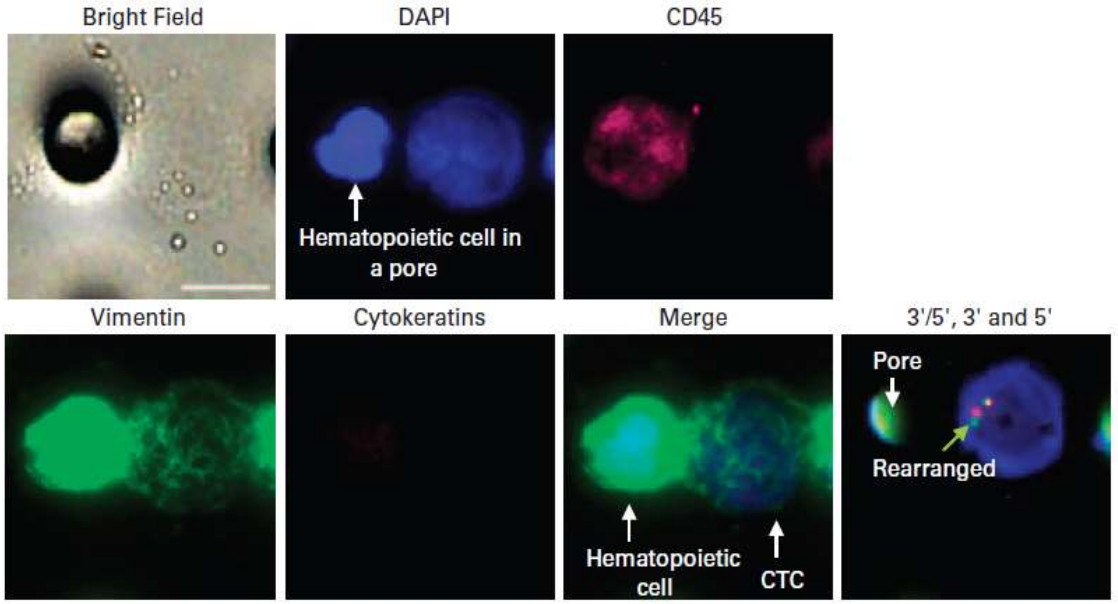
- CTCs isolated by ISET from 87 patients with lung adenocarcinoma
- ALK break-apart FISH (Abbott)
- anti-ALK antibody (5A4 clone)
- Blindly on CTCs and corresponding tumor tissues

- Five patients positive for ALK-gene rearrangement and strong ALK ICC in CTCs and T tissue.
- 82 patients negative for both ALK FISH and ALK ICC in CTCs and T tissue

JOURNAL OF CLINICAL ONCOLOGY ORIGINAL REPORT

Detection of Circulating Tumor Cells Harboring a Unique *ALK* Rearrangement in *ALK*-Positive Non–Small-Cell Lung Cancer

Emma Pailler, Julien Adam, Amélie Barthélémy, Marianne Oulhen, Nathalie Auger, Alexander Valent, Isabelle Borget, David Planchard, Melissa Taylor, Fabrice André, Jean Charles Soria, Philippe Vielh, Benjamin Besse, and Françoise Farace



Representative example of vimentin/cytokeratins/ CD45/DAPI immunofluorescent staining of ALK-rearranged CTCs in a ALK-positive patient

18 ALK-positive patients
14 ALK-negative patients
100 % consistant result (T, CTC)
100% of patients with detectable CTCs by ISET

Single cell analysis: ALK-rearranged CTCs expressed a mesenchymal phenotype contrasting with heterogenous epithelial and mesenchymal marker expressions in tumors

CTC challenges

Without decreasing the sensitivity
and keeping cells with intact morphology

CTC isolated unfixed for NGS, RNA analysis, culture, injecton into mice

CTC collection on a slide: high throughput immuno and FISH analysis

CTC stabilization and long storage for days

ISET* by Rarecells & CellCelector™ by ALS: powerful enrichment & selection of circulating rare cells

Blood drawing

Drawing a 10 mL-blood sample

Filtration

Dilution with Rarecells buffer for unfixed cells
Filtration by Rarecells® Device (3 min)

Sample preparation

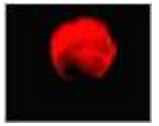
Bright field or Immune Labeling : ex: EpCAM or Cytokeratin or Vimentin, etc..

Cell selection and picking

Very Fast scanning, cell detection, selection and picking with CellCelector™

Single-cell genetic characterization

BF



CK

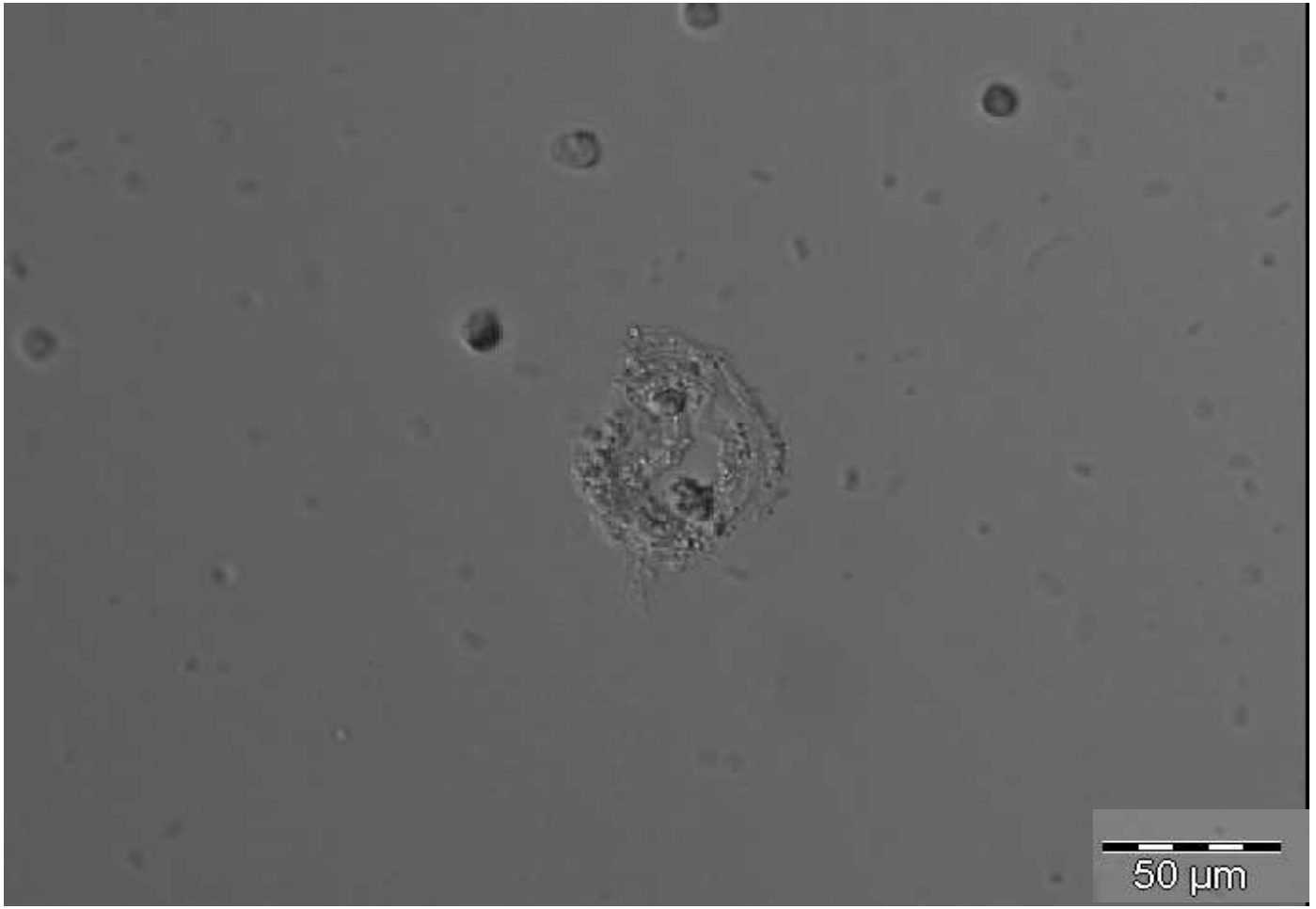
- WGA, genotyping, aneuploidy detection, mutation panel detection
- Similar workflow to PGD

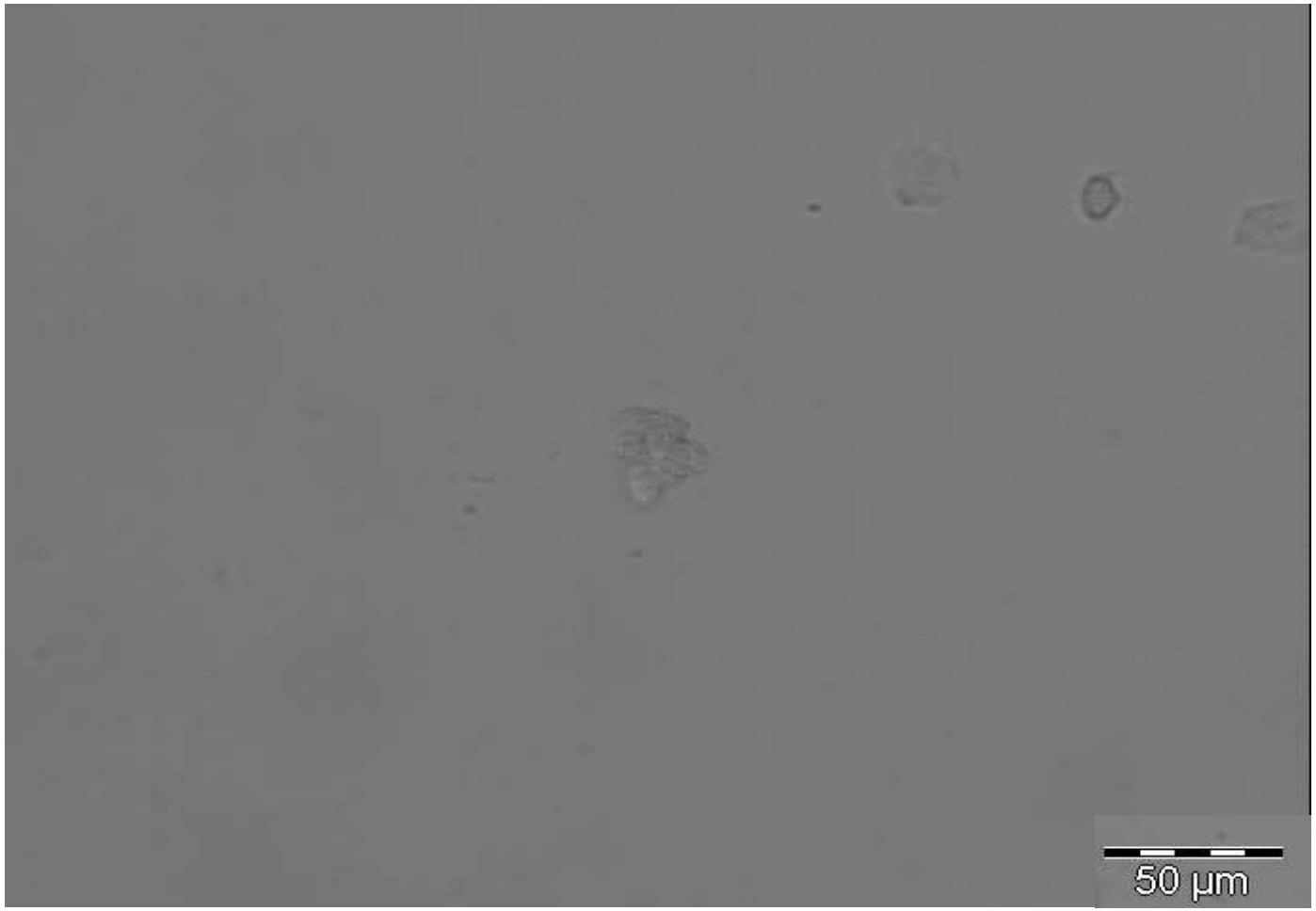
* ISET: Isolation by SizE of Tumor/Trophoblastic Cells

Sensitivity of isolation unfixed CRC

Number of micropipetted cells in 1 mL of blood	Number of cells detected (A549)	Recovery (A549)
1	1	100%
	1	100%
	1	100%
	1	100%
	1	100%
	1	100%
	1	100%
3	3	100%
4	3	75%
5	4	80%
	5	100%
	4	80%
	4	80%
	3	60%
	5	100%
10	9	90%
	8	80%
	9	90%
	10	100%
	8	80%
Overall recovery		**91%**

Unpublished data


50 µm


50 µm

Whole genome amplification methods

	PEP	MDA RepliG (Qiagen) Genomiphi (GE)	GenomePlex (Sigma)	PicoPlex (Rubicon genomics)	MALBAC (Yikon Genomics)
Time	8 h (o/n)	2 h /8 h	~ 4 h	2 h	4 h
Cost per reaction	2,6 €	5,24 € (Qiagen) 3,88 € (GE)	12,7 €	28 $	34,3 €
Yield	ng range?	10 µg / 40 µg from 10 ng purified gDNA	5-10 µg (fresh single cell)	2-5 µg (fresh single cell)	3 µg (fresh single cell)
Proportional	-	-	+	++	Better than MDA
Genome coverage in HTS (fresh single cell)		72% of the genome at ≥1x coverage (25x seq depth) (Zong et al, 2012)	6% of the genome at 0,2x coverage (Navin et al, 2011)	unpublished	85 to 93 % of the genome at ≥1x coverage (25x seq depth) (Zong et al, 2012)

Feasibility of single cell NGS by whole exome sequencing (Illumina)

Sample type	number of reads	number of mapped reads	Percentage of coverage	Average sequencing depth
Unfixed (Fresh) Single cell	51 600 412	45 5341 717	76 %	27
Fixed single cell	40 769 194	36 255 925	70 %	22
Fixed and microdissected single cell	38 455 158	32 076 608	50 %	20
Genomic DNA	43 827 640	35 532 707	95 %	23

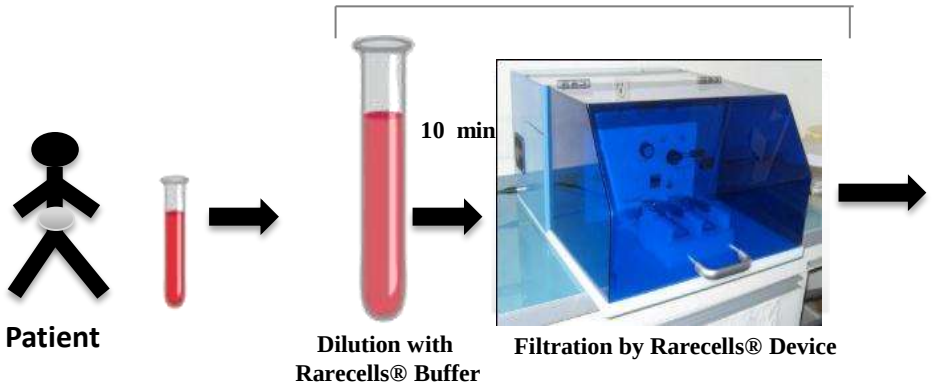
In collaboration with FASTERIS

Rarecells

CTC transfer on slides

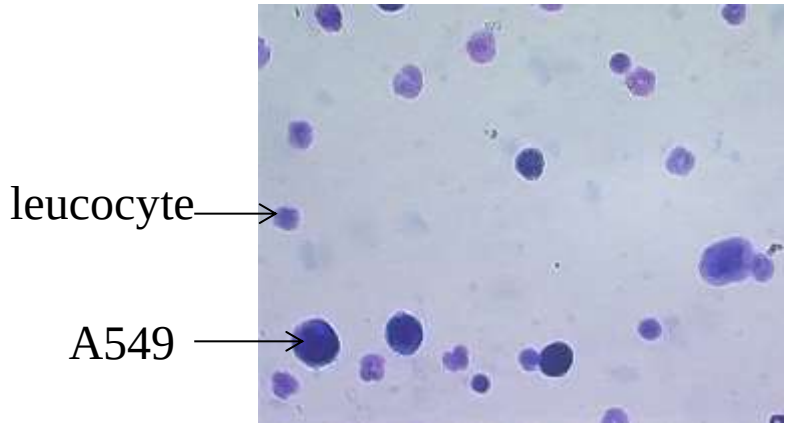
Step 1 –
Blood Collection
10 mL

Step 2- Filtration with Rarecells® System (15 min)
Blood to be processed within 5 hours of collection



Step 3- Transfer on slide

Step 4
Staining and cytopathology



Ongoing: sensitivity validation (1 tumor cell per mL of blood)

VHL mutation analysis in CTCs from patients with Clear Cells Renal Carcinoma

30 patients with CCRC

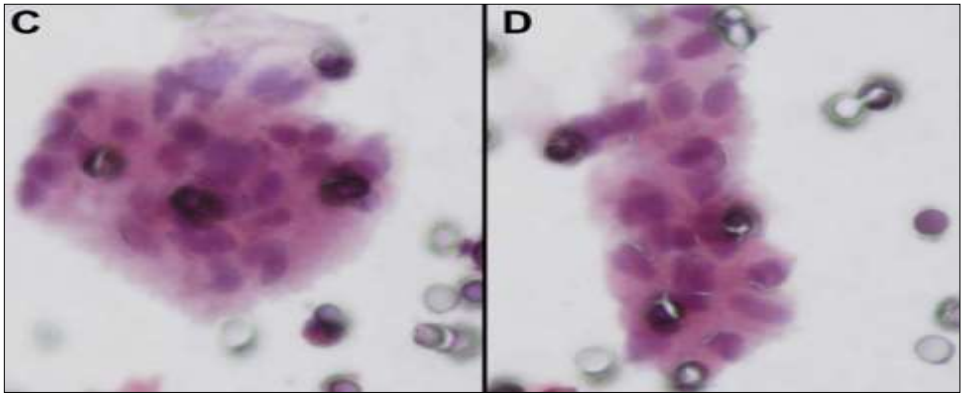
22 men et 8 women

Mean age 68,5

Two patients with metastasis

Tested by ISET before surgery

Mutation analysis: T-CTC- gDNA



CTM from CCRC patients

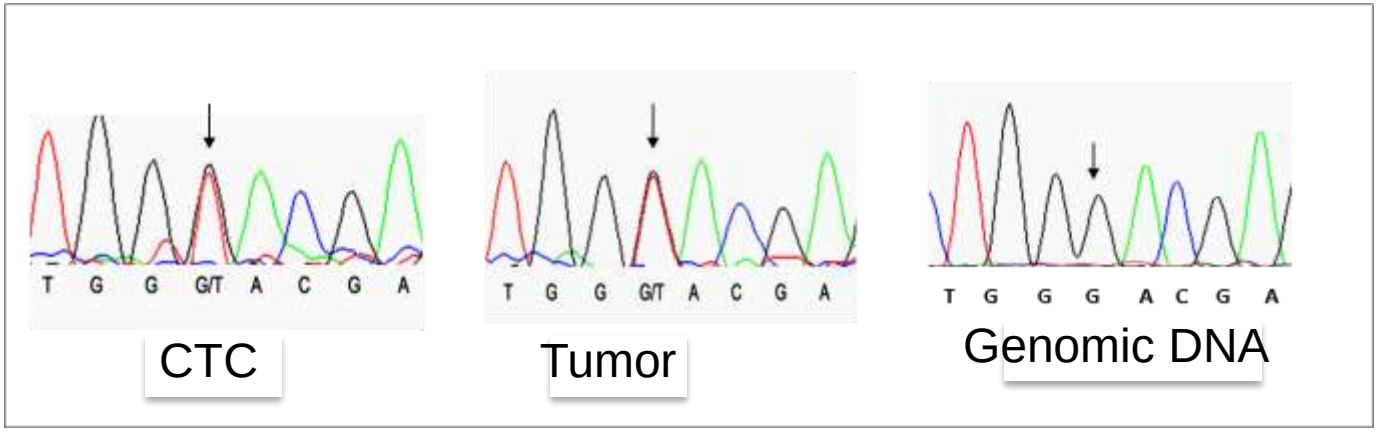
- VHL gene
- Identified in 1993 by Latif et al.
- Tumor suppressor gene
- Position: 3p25.26 - 20Kb – 3 exons
- 2 ARNm de 4,5 et 4,7 Kb, two protein isoforms
- More than 500 mutations

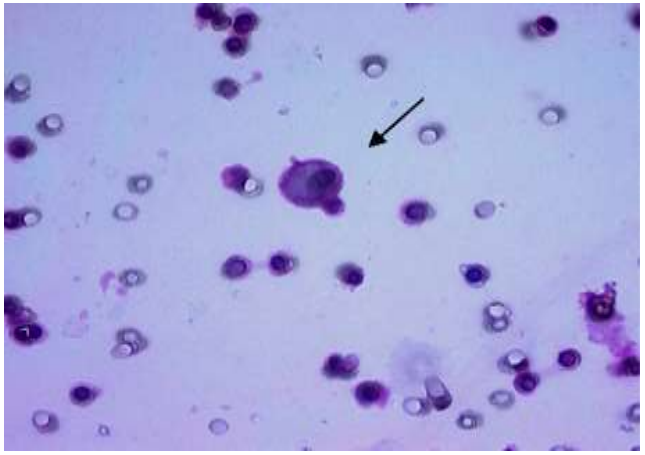
VHL mutations found

EXON	CODON	MUTATION	TYPE OF MUTATION	CODON INITIAL	CODON MUTE	CpG	PROTEIN CHANGE
1	9	c.27G>T	Tr	GAC	TAC	Oui	D9Y
	18	c.53C>A	Tr	GCA	GAA	Non	A18E
	61	c.183C>G	Tr	CCC	CCG	Non	P61P
	65	c.194C>A	Tr	TCG	TAG	Non	S65X
	69	c.205-206delCG	Fr	CGC	-	-	E69X
	88	c.263G>A	Ts	TGG	TAG	Non	W88X
	92	c.275delA	Fr	GAC	-	-	D92X
	100	c.299delC	Fr	ACG	-	-	T100X
	109	c.327delC	Fr	ATC	-	-	H109X
2	116	c.346C>G	Tr	CTT	GTT	Non	L116V
	118	c.353T>C	Ts	CTC	CCC	Non	L118P
	140	c.418delC	Fr	CTC	-	-	L140X
	145	c.435G>T	Tr	CAG	CAT	-	Q145H
3	158	c.472delC	Fr	CTG	-	-	L158X
	163	c.486delC	Fr	TGC	-	-	L163X
	176	c.526A>T	Tr	AGG	TGG	Non	R176W
	183	c.548C>A	Tr	TCG	TAG	Non	S183X
	207	c.620C>T	Ts	GCA	GTA	Non	A207V

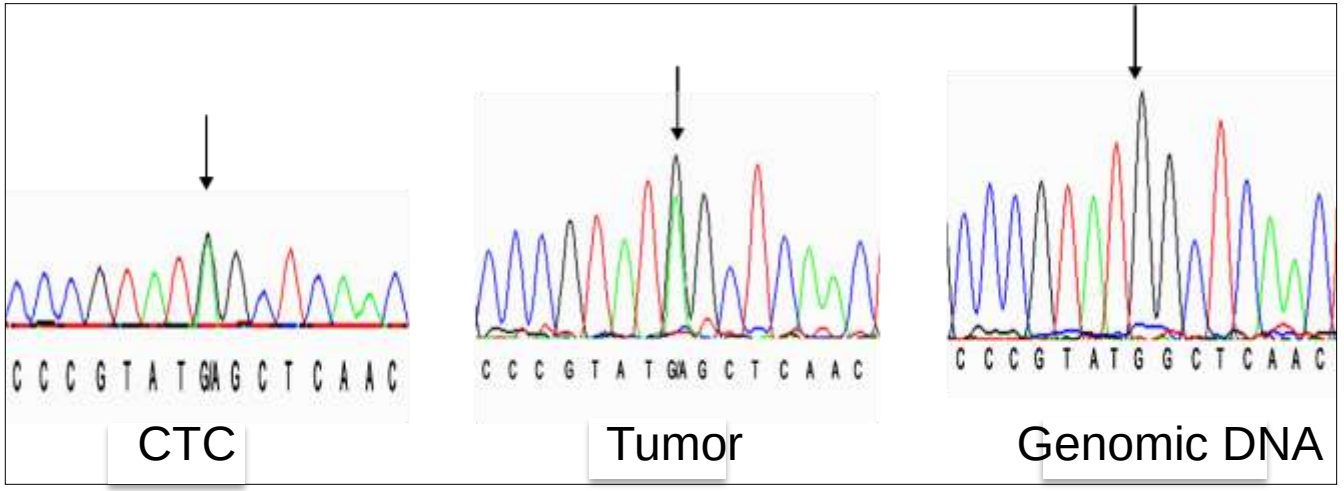
Tr : Transversion; Ts : Transition; Fr : Frameshift

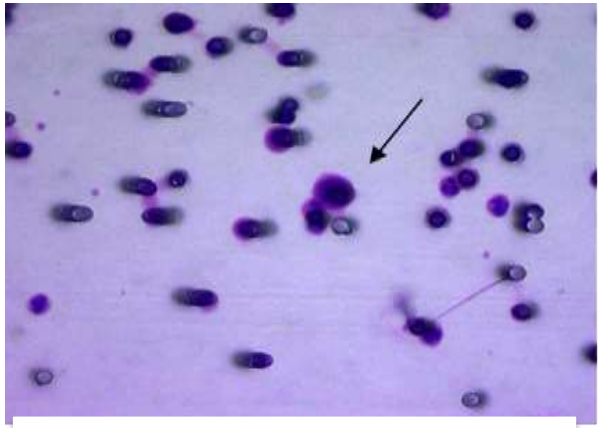
- **Exemple of transversion : Mutation c.27G>T, codon 9 / Exon 1 (1 patient)**



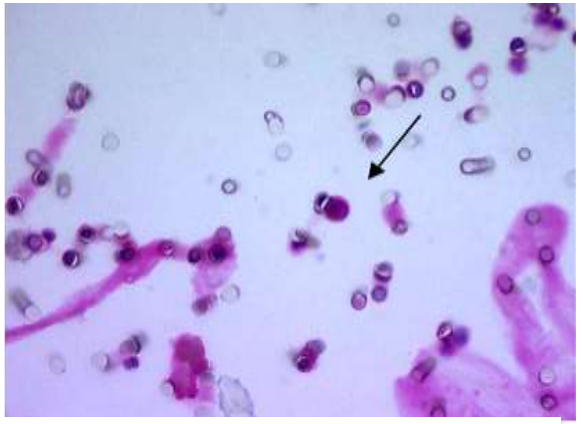


Exemple of a transversion found in 2 patients
Mutation c.263G>A, codon 88 / Exon 1





CTC from patient CGN



CTC from patient GRA

VHL mutation analysis can help cytopathological diagnosis of CTCs in patients with Clear Cells Renal Carcinoma

Blind mutation analysis of VHL gene in T, leucocytes and CTC isolated by ISET

Cytoapthology

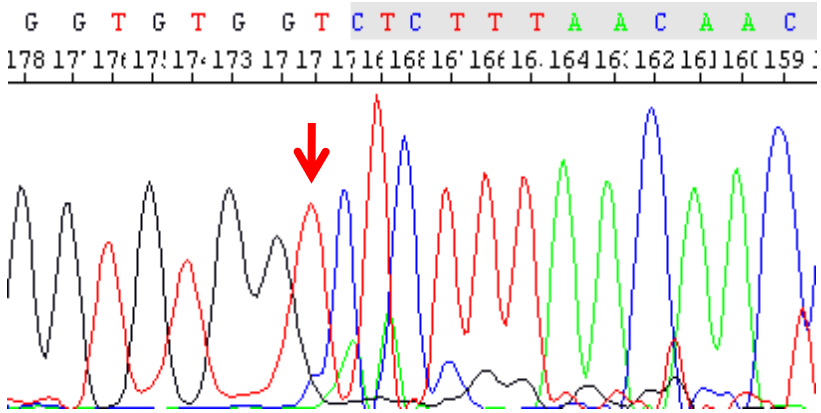
30 patients with CCRC
25 with VHL mutation in tumor

20 patients with CTC

205 single cell analysis : 64 CTC

VHL mutations: 57/64 CTC

7 VHL-neg CTC from tumors neg for VHL



VHL gene exon 2: T insertion in position 171
Mutation: GGTGTGGTCTCTTTAA
(WT: GGTGTGGCTCTTTAA)

Ben Njima B et al, manuscript in preparation

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