FUTURE PERSPECTIVES OF CIRCULATING TUMOR DNA IN COLORECTAL CANCER

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SELECTED HIGHLIGHTS

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KEY MESSAGES

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- Liquid biopsy using ctDNA analysis has the potential to revolutionise colorectal cancer management with roles in:
  - Cancer diagnosis
  - Detection of tumor recurrence or minimal residual disease and determining prognosis
  - Tracking resistance and tailoring therapies
- The key benefit is the ability to obtain a dynamic, real-time picture of the tumor genomic landscape in a given patient, providing the opportunity to tailor therapies throughout the disease course, from diagnosis to the development of resistance
- Application of this technology to detect minimal residual disease and to monitor the emergence of molecular resistance have a high clinical relevance
- Large prospective trials are needed, and it is essential to standardise the techniques used to analyse ctDNA,

LIQUID BIOSPY: RATIONALE

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- Resistance to targeted therapy develops over time due to tumor heterogeneity, clonal evolution and selection[1]
- Tumor biopsy is the current gold standard for both diagnosis and monitoring of resistance, but has significant drawbacks including:
  - Difficulties obtaining sufficient tumor material for analysis
  - The need for invasive serial biopsies
  - Sampling bias due to tumor heterogeneity[2-4]
- "Liquid biopsy" is the common term for characterising the genetic profile of a tumor based on a blood sample

1. Greaves M and Maley CC. Nature 2012; 481: 306-13. 2. Bedard PL, et al. Nature 2013; 501: 355-63. 3. Burrell RA, et al. Nature 2013; 501: 338-45. 4. Meacham CE and Morrison SJ. Nature 2013; 501: 328-37.

COMPARISON OF CTCs VERSUS cfDNA

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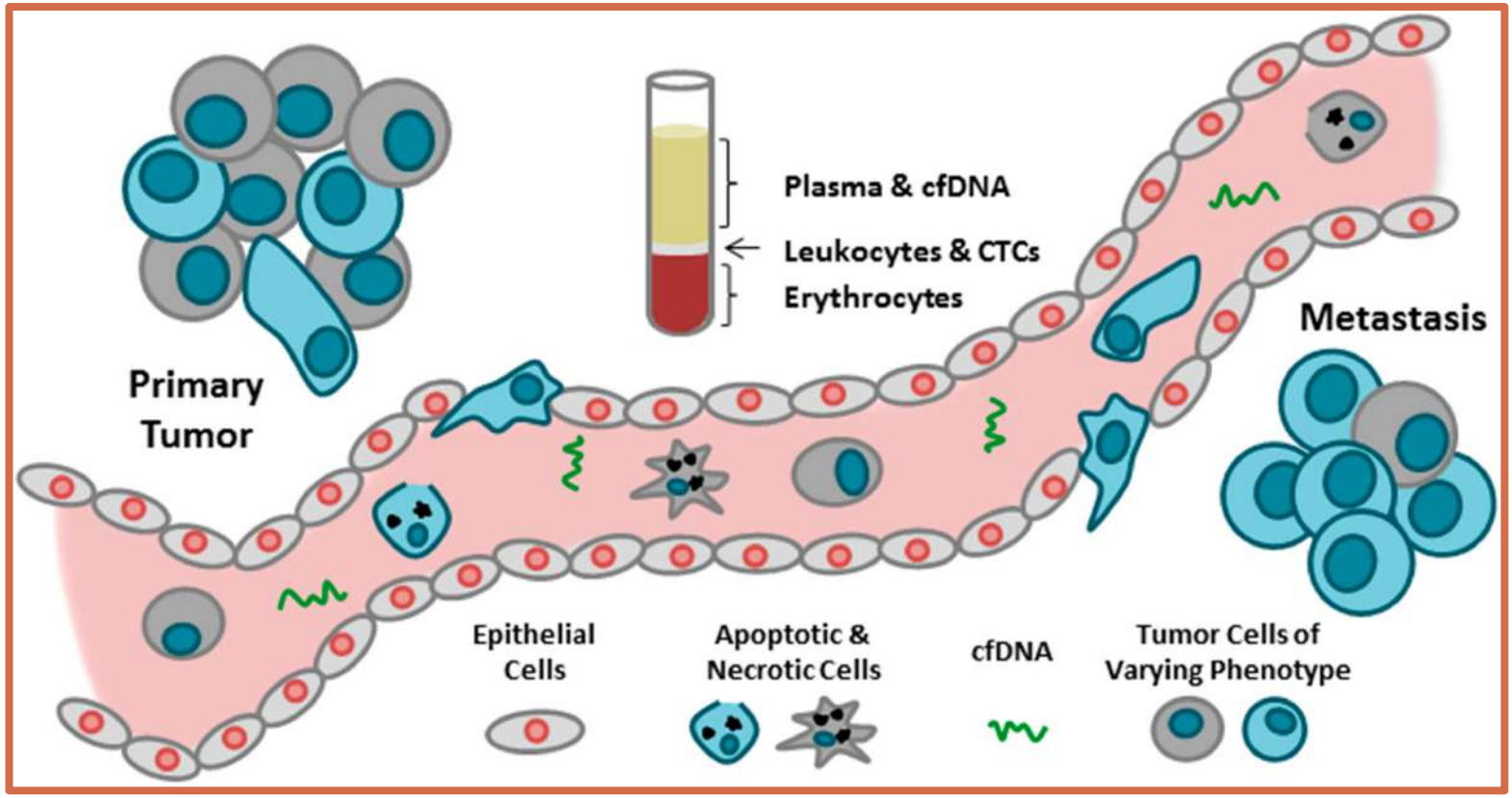

CTCs and cfDNA have been demonstrated to provide prognostic information[1]

1. Lowes, LE, et al. Int J Mol Sci. 2016; 17: [Epub].

LIQUID BIOPSY: CIRCULATING TUMOR CELLS OR DNA?

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- Unlike ctDNA, circulating tumor cells (**CTCs**) offer the ability to gain insight into the characteristics of cells responsible for metastasis[1]
- However, CTCs occur at very low levels in blood (1 in 10^6 to 10^7 nucleated blood cells), presenting a challenge for detection[2]
  - Some patients test positive for ctDNA and negative for CTCs[3]
  - CTC counts are lower in CRC than in other cancers – CTCs released from a tumor in the colon may become trapped in the liver before reaching the systemic circulation[4]
- Using current techniques, the diagnostic performance of ctDNA is superior to that of CTCs in CRC

1. Gazzaniga P, et al. Expert Rev Mol Diagn. 2015; 15: 925-32. 2. Yang D, et al. Mol Cell Ther. 2014; 2: 8.
3. Bettegowda C, et al. Sci Transl Med. 2014; 6: 224ra24. 4. Gkountela S, et al. ESMO Open. 2016; 1: e000078.

LIQUID BIOPSY: REAL-TIME MONITORING IS FEASIBLE WITH ctDNA ANALYSIS

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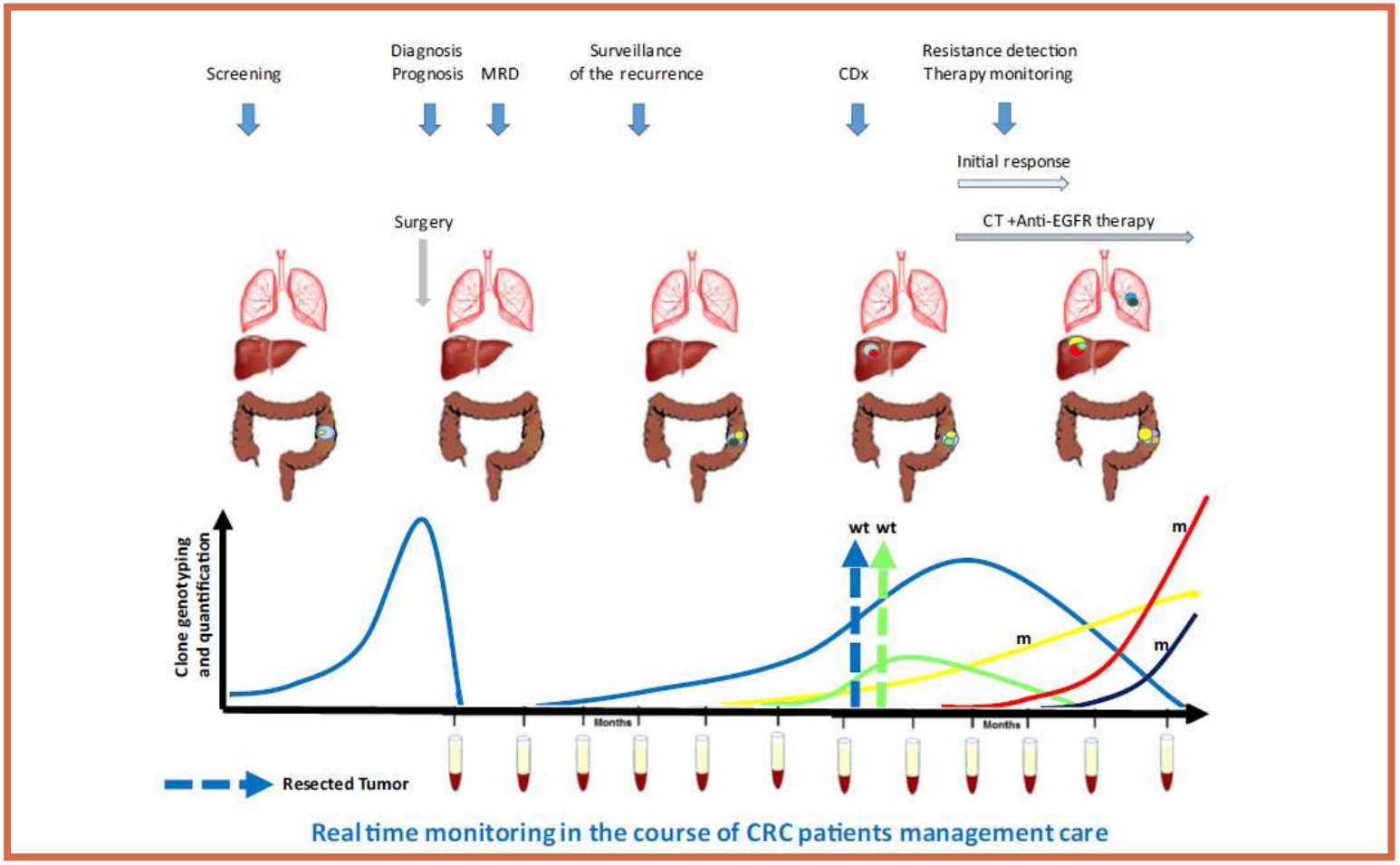

Real time monitoring in the course of CRC patients management care

1. Thierry AR, et al. Cancer Metastasis Review 2016; 35: 347-76.

SCOPE OF THE REVIEW

- This review focuses on the potential clinical applications of liquid biopsy using circulating tumor DNA (**ctDNA**) in colorectal cancer (CRC), including its role:
  - Diagnosis and screening
  - Determining prognosis
  - Monitoring tumor burden
  - Monitoring response
  - Evaluating resistance during treatment
  - Detecting recurrence

TUMOR DNA FOR LIQUID BIOPSY: POTENTIAL SOURCES

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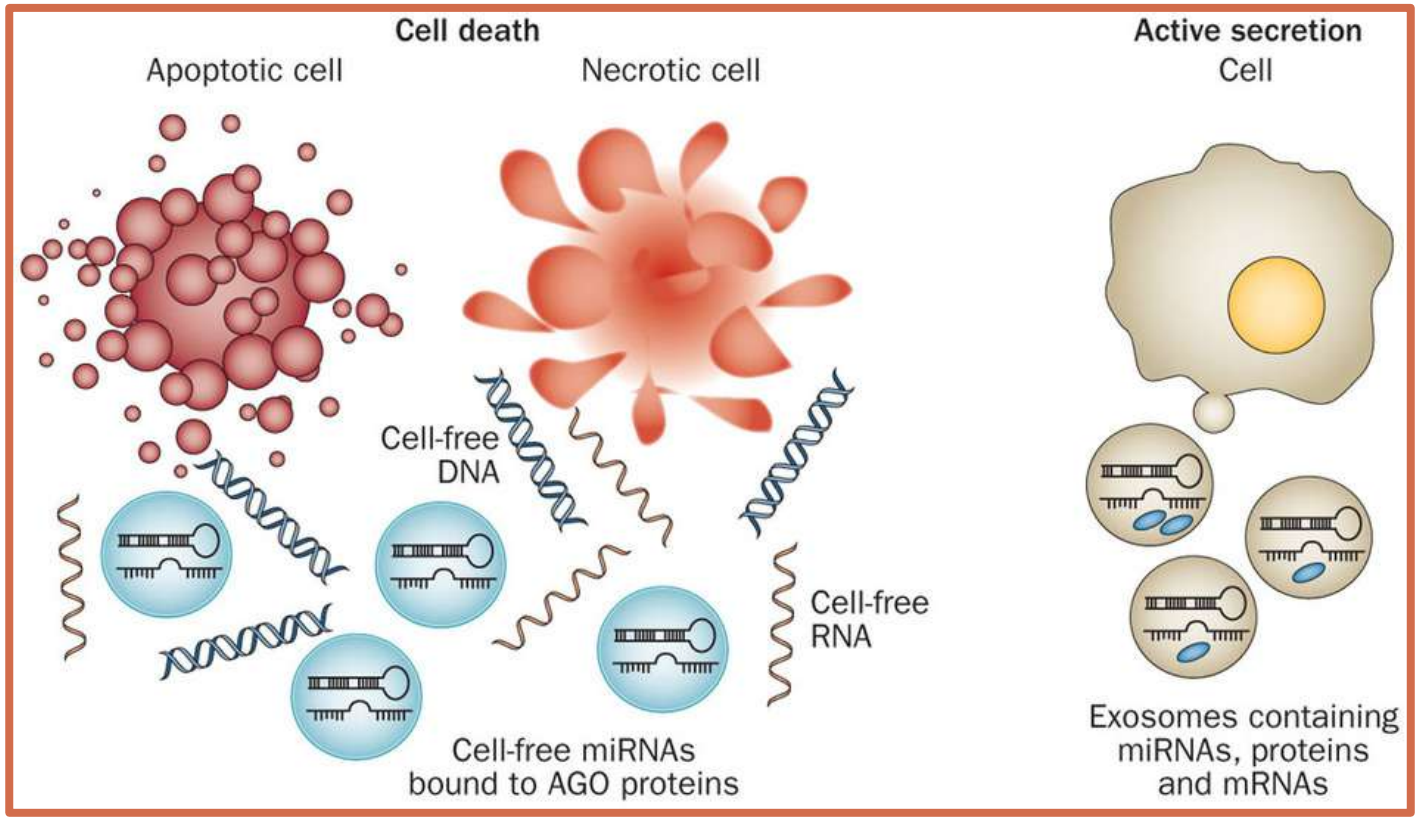
- Cancer patients have higher plasma levels of cell-free DNA (**cfDNA**) than healthy individuals, although cfDNA elevation is not specific to cancer[1]
- ctDNA is the fraction of cfDNA that carries tumor-specific alterations[2]
  - Apoptosis is the major mechanism of ctDNA release[2,3]
  - Plasma is the preferred source of ctDNA for liquid biopsy, as serum ctDNA is diluted by genomic DNA released from white blood cells[4,5]
- Tumor-derived **exosomal DNA** is promising as a future alternative to ctDNA[6,7] (tumor-derived genomic material fro extracellular vesicles is more concentrated and better preserved as compared ctDNA)
- Analysis of circulating free or exosomal **microRNA** (miRNA) is also under investigation (see next slide)[8]

1. Leon SA, et al. Cancer Res. 1977; 37: 646-50. 2. Jahr S, et al. Cancer Res. 2001; 61: 1659-65. 3. Stroun M, et al. Clin Chim Acta. 2001; 313: 139-42. 4. Diehl F, et al. Proc Natl Acad Sci U S A. 2005; 102: 16368-73. 5. Holdhoff M, et al. J Natl Cancer Inst. 2009; 101: 1284-5. 6. Cai J, et al. Exp Cell Res. 2016; 349: 179-83. 7. Gold B, et al. J Mol Diagn. 2015; 17: 209-24. 8. Uratani R, et al. PLoS One 2016; 11: e0160722.

POTENTIAL ROLE OF miRNAs IN DIAGNOSIS, PROGNOSIS AND ASSESSING RESPONSE



miRNAs are released from cells into blood, which then circulate in various secreted extracellular vesicles, such as apoptotic bodies and exosomes[1]

AGO, Argonaute

1. Schwarzenbach H et al. Nat Rev Clin Oncol 2014;11:145-56.

DETECTING ctDNA: ADVANCES IN SEQUENCING TECHNIQUES

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Principle of detection	Techniques	Sensitivity	Application	Limitations	Advantages	Clinical use with ctDNA
Digital PCR	BEAMing, Droplet-based digital PCR, Microfluidic digital PCR	0.01%-0.1%	SNV, known genomic rearrangements only	Specific equipment, cost	High sensitivity	Recurrence, prognosis, monitoring response and resistance
Targeted deep sequencing	Safe-SeqS, TAmSeq, Ion-AmpliSeq CAPP-Seq	0.01%-0.1%	SNV, CNV, rearrangements across targeted regions only	PCR sampling bias and sequencing errors	High sensitivity, cost decreasing	Diagnosis, screening, prognosis
Whole-genome sequencing	PARE, Whole-exome sequencing	1%	Genome-wide SNV, CNV, rearrangements	Expensive, sensitivity improvement ongoing	Genome-wide applications	*Future:* diagnosis, screening, tracking resistance

BEAMing, beads, emulsion, amplification, and magnetics; CAPP-Seq, cancer personalized profiling by deep sequencing; CNV, copy number variation; PARE, personalized analysis of rearranged ends; PCR, polymerase chain reaction; Safe-SeqS, safe-sequencing system; SNV, single nucleotide variation; TamSeq, tagged-amplicon deep sequencing.

SCREENING AND DIAGNOSIS: METHYLATED ctDNA

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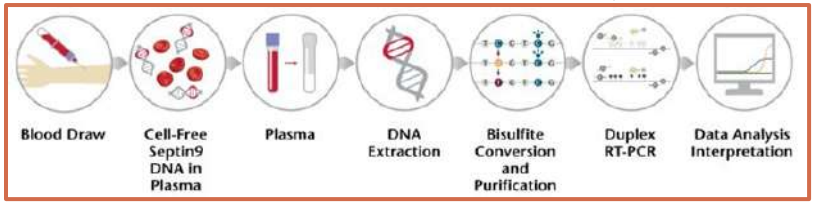
- ctDNA detection of individual mutations such as *KRAS* and *BRAF* V600E has insufficient sensitivity for population screening (unmutated tumors are undetectable)
- Analysing aberrant DNA methylation of specific gene promoter regions in ctDNA is an alternative approach[1]
- Methylated *SEPT9* has been evaluated in a number of CRC screening studies, with mixed results[2,3] (sensibility from 50 to 90% mostly dependant of tumor stage)
    - A *SEPT9* methylation assay is approved in the US for screening of CRC (*Epi proColon®; Epigenomics, Inc., Germantown, MD*)



- A *BCAT1* and *IKZF1* methylation assay identified ~70% of CRC in recent studies[4-6]

1. Warton K, et al. Endocr Relat Cancer. 2016; 23: R157-71. 2. Church TR, et al. Gut 2014; 63: 317-25. 3. Yan S, et al. Med Sci Monit. 2016; 22: 3409-18. 4. Pedersen SK, et al. BMC Cancer. 2015; 15: 654. 5. Symonds EL, et al. Clin Transl Gastroenterol 2016; 7: e137. 6. Young GP, et al. Cancer Med 2016; 5: 2763-72.

SCREENING AND DIAGNOSIS: MULTIGENE PANELS

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- Multigene methylation panels may improve sensitivity and specificity[1-5]

- A multi-target, stool-based CRC screening test (*Cologuard*®)[6] is also approved in the US
- Nevertheless, further data and standardisation are needed before these approaches are ready for clinical practice

1. Cassinotti E, et al. Int J Cancer. 2012; 131: 1153-7. 2. Lee BB, et al. Clin Cancer Res. 2009; 15: 6185-91.
3. Pedersen SK, et al. BMC Cancer. 2015; 15: 654. 4. Symonds EL, et al. Clin Transl Gastroenterol 2016; 7: e137.
5. Young GP, et al. Cancer Med. 2016; 5: 2763-72. 6. Imperiale TF, et al. N Engl J Med. 2014; 370: 1287-97.

PROGNOSTIC VALUE OF ctDNA

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- Many CRC patients receive potentially toxic and unnecessary adjuvant therapy due to an inability to identify which patients will experience disease recurrence after surgery

- Detection of ctDNA before surgery has been linked to a high risk of recurrence[4,7-12] and shorter disease-free survival and overall survival.[12-15]

- Detection of ctDNA after surgical resection is associated with minimal residual disease and a high recurrence rate (>90%) which can be detect before radiologic recurrence (up to 10 months before)[1,2-6, 17]

- Large trials are needed to confirm the prognostic value of ctDNA

1. Diehl F, et al. Nat Med. 2008; 14: 985-90. 2. Mouliere F, et al. PLoS One. 2011; 6: e23418. 3. Ryan BM, et al. Gut 2003; 52: 101-8.
4. Bazan V, et al. Ann Oncol. 2006; 17(Suppl 7): vii84-90. 5. Frattini M, et al. Cancer Lett. 2008; 263: 170-81. 6. Tie J, et al. Sci Transl Med. 2016; 8: 346ra92. 7. Wang JY, et al. World J Surg. 2004; 28: 721-6. 8. Lee HS, et al. Transl Oncol. 2013; 6: 290-6. 9. Hsieh JS, et al. Am Surg. 2005; 71: 336-43. 10. Nakayama G, et al. Anticancer Res. 2011; 31: 1643-6. 11. Nishio M, Anticancer Res 2010; 30: 2673-82. 12. Lecomte T, et al. Int J Cancer. 2002; 100: 542-8. 13. Leung WK, et al. Am J Gastroenterol. 2005; 100: 2274-9. 14. Wallner M, et al. Clin Cancer Res. 2006; 12: 7347-52. 15. Trevisiol C, et al. Int J Biol Markers. 2006; 21: 223-8. 16. Ito S, Jpn J Cancer Res. 2002; 93: 1266-99.
17. Reinert T, et al. Gut 2016; 65: 625-34.

DETECTING RECURRENCE AFTER CURATIVE SURGERY USING ctDNA

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Reference	Gene(s)	Treatment	Patients (N)	Detection rate of recurrence in plasma, %*
Diehl 2008[1]	*APC, TP53, KRAS, PI3K*	Surgery ± chemotherapy (61%)	18	100% (15/15)
Frattini 2008[2]	Total DNA, *KRAS, p16INK4a*	Surgery ± chemotherapy	70	100% (18/18)
Ryan 2003[3]	*KRAS*	Surgery ± chemotherapy (53%)	94	91% (10/11)
Tie 2016[4]	Personalised assay	Surgery ± chemotherapy	230	41% (11/27) in surgery-only group

* (n positive ctDNA / n recurrence)

1. Diehl F, et al. Nat Med 2008; 14: 985-90; 2. Frattini M, et al. Cancer Lett. 2008; 263: 170-81;
3. Ryan BM, et al. Gut 2003; 52: 101-8; 4. Tie J, et al. Sci Transl Med. 2016; 8: 346ra92.

MONITORING TUMOR BURDEN AND RESPONSE ON TREATMENT

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- Serial plasma ctDNA measurements can track tumor burden dynamics over time, outperforming carcino-embryonic antigen (CEA)[1]

- Liquid biopsy also has potential for monitoring treatment response (see next slide)[2-5]

- Changes in ctDNA levels may predict treatment response early in the course of therapy, thereby creating a critical window of opportunity for intervention (i.e. treatment modification)

1. Diehl F, et al. Nat Med. 2008; 14: 985-90. 2. Reinert T, et al. Gut 2016; 65: 625-34.
3. Lefebure B, et al. Ann Surg 2010; 251: 275-80. 4. Spindler KL, et al. Int J Cancer 2014. 135: 2215-22; 5. Spindler KL, et al. Clin Cancer Res 2012; 18: 1177-85.

ctDNA AND TREATMENT OUTCOMES

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Reference	Gene(s)	Treatment	Patients (N)	Detection rate in plasma, %*	Predictive value
Lefebure 2010[1]	*KRAS*, *RASSF2A* methylation	Chemotherapy	31	52% (12/23)	**Shorter PFS in patients with positive ctDNA** (median 5 vs 14 months; *P*=0.004).
Spindler 2014[2]	Total cfDNA and *KRAS*	Cetuximab + irinotecan	73	–	**Higher response rate in patients with >50% decrease in total cfDNA at cycle 3** (40% vs 17%; *P*=0.04)† **Poor disease control in patients with high levels of *KRAS*-mutant ctDNA** (0% vs 42%; *P*=0.048)‡
Spindler 2012[3]	Total cfDNA and *KRAS*	Cetuximab + irinotecan	108	78% (32/41)	**Low baseline cfDNA associated with higher disease control rate**

* (n plasma positive / n tumors positive); † Versus patients with <50% decrease in cfDNA; ‡ Disease control rate for patients with *KRAS*-mutant ctDNA in the highest quartile vs those with lower levels.
PFS, progression-free survival.

1. Lefebure B, et al. Ann Surg 2010; 251: 275-80; 2. Spindler KL, et al. Int J Cancer 2014; 135: 2215-22; 3. Spindler KL, et al. Clin Cancer Res 2012; 18: 1177-85.

cfDNA LEVEL HAS A STRONG PROGNOSTIC VALUE IN CRC

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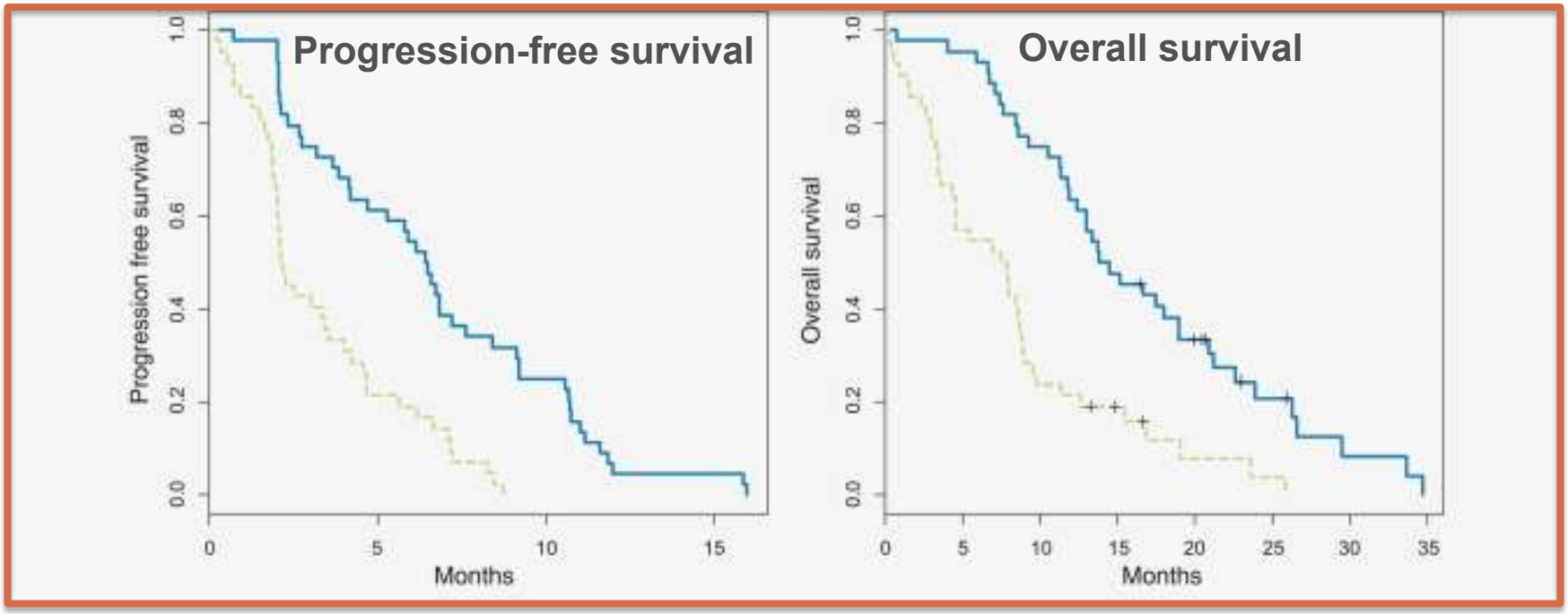

Progression-free and overall survival for patients stratified by cfDNA low* (blue line) and high* (dashed line) as reported by Spindler et al.[1]

*Threshold of 7,100 alleles/mL

1. Spindler K-L et al. Int J Cancer. 2014;135:2984-52991.

TRACKING RESISTANCE AND TAILORING THERAPY

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- During targeted therapy, serial monitoring of ctDNA may enable early detection of molecular changes that confer resistance, providing an opportunity for treatment modification
- A cornerstone for ctDNA analysis in CRC has been the identification of emerging *RAS* mutations for acquired resistance to anti-EGFR therapy[1-4]
  - Numerous secondary *KRAS* mutations have been identified in serum ctDNA and may be detected 5-10 months before radiologic documentation of disease progression[1,3,4]
- Additional mechanisms of secondary resistance detected in ctDNA during anti-EGFR therapy include up to 70 novel mutations in MAPK pathway genes,[5] and *MET* amplification[6]

1. Misale S, et al. Nature 2012;486:532-536; Spindler KL, et al. Int J Cancer 2014; 135: 2215-2222.
3. Diaz LA, Jr., Bardelli A. J Clin Oncol 2014; 32: 579-586; 4. Diaz LA, Jr., et al. Nature 2012; 486: 537-540; 5. Bettegowda C, et al. Sci Transl Med 2014;6:224ra224; 6. Bardelli A, et al. Cancer Discov 2013;3:658-673.

KRAS MUTATIONS AND ACQUIRED RESISTANCE TO ANTI-EGFR THERAPY

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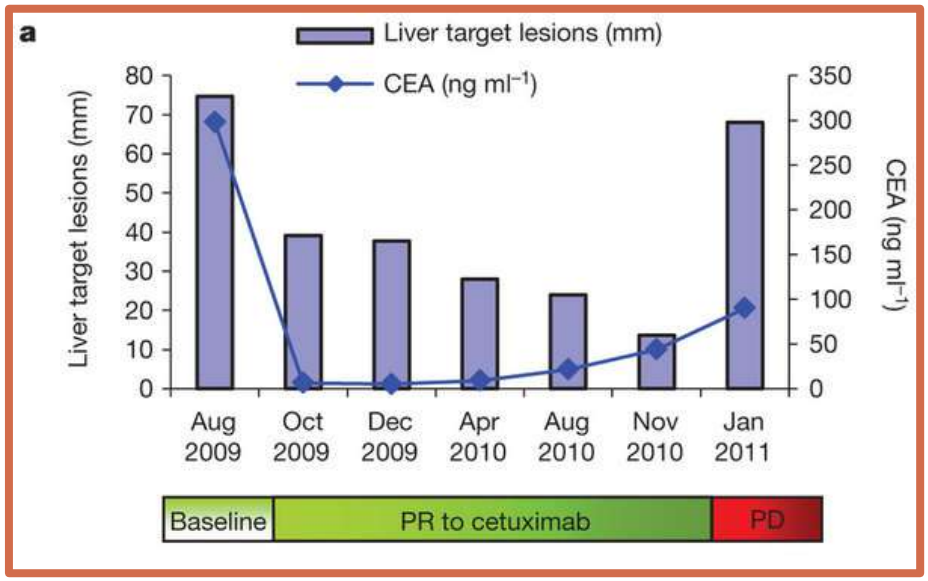

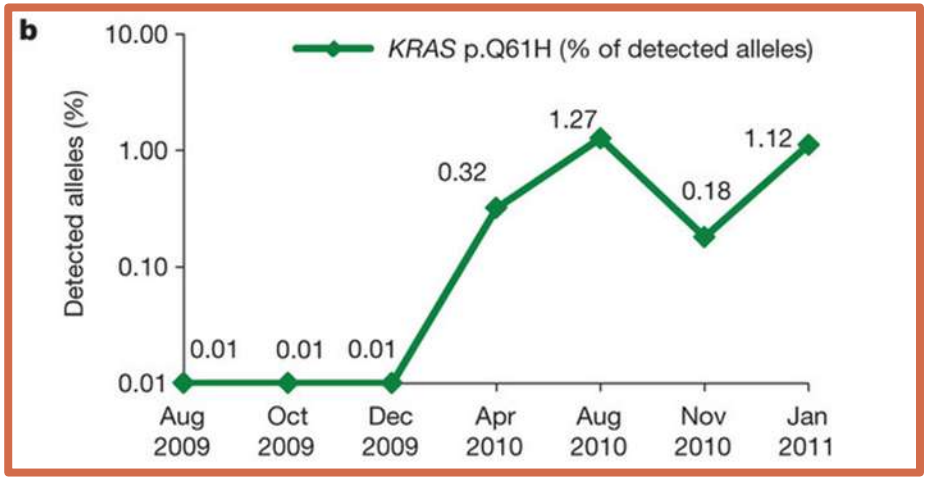

Detection of circulating *KRAS* mutant DNA in a patient with acquired resistance to cetuximab therapy as reported by Misale et al.[1]

1. Misale S, et al. Nature. 2012; 486: 532-6.

CONCLUSION

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- Large, well-designed trials using standardized methodology are needed to:
  - Identify the most sensitive biomarkers for CRC screening
  - Compare relevance of ctDNA mutation vs tissue at diagnostic but also to track resistance.
  - Validate clinical relevance of ctDNA to evaluated tumor burden (i.e. change earlier chemotherapy regimen if ctDNA increase before radiologic progression is associated with an increased survival ?)
  - Compare the performance of ctDNA with CTCs and, in future, extracellular vesicles
- Transition of blood-based assays from bench to bedside is ongoing


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