FUTURE PERSPECTIVES OF CIRCULATING TUMOR DNA IN COLORECTAL CANCER

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SELECTED HIGHLIGHTS

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KEY MESSAGES

• 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

• 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

CTCs and cfDNA have been demonstrated to provide prognostic information\textsuperscript{1}

LIQUID BIOPSY: CIRCULATING TUMOR CELLS OR DNA?

• 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

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

Real time monitoring in the course of CRC patients management care

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

- 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 from extracellular vesicles is more concentrated and better preserved as compared to ctDNA)
- Analysis of circulating free or exosomal **microRNA** (miRNA) is also under investigation (see next slide)\(^8\)

miRNAs are released from cells into blood, which then circulate in various secreted extracellular vesicles, such as apoptotic bodies and exosomes\(^1\)

AGO, Argonaute

# DETECTING ctDNA: ADVANCES IN SEQUENCING TECHNIQUES

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

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

- 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
  - Methylated SEPT9 has been evaluated in a number of CRC screening studies, with mixed results (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

SCREENING AND DIAGNOSIS: MULTIGENE PANELS

- Multigene methylation panels may improve sensitivity and specificity\(^1-5\)

- A multi-target, stool-based CRC screening test (*Cologuard*\(^\text{®}\)\(^6\)) is also approved in the US

- Nevertheless, further data and standardisation are needed before these approaches are ready for clinical practice

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PROGNOSTIC VALUE OF ctDNA

- 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\textsuperscript{4,7-12} and shorter disease-free survival and overall survival.\textsuperscript{12-15}

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

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

## DETECTING RECURRENT AFTER CURATIVE SURGERY USING ctDNA

<table>
<thead>
<tr>
<th>Reference</th>
<th>Gene(s)</th>
<th>Treatment</th>
<th>Patients (N)</th>
<th>Detection rate of recurrence in plasma, %*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diehl 2008¹</td>
<td><em>APC, TP53, KRAS, PI3K</em></td>
<td>Surgery ± chemotherapy (61%)</td>
<td>18</td>
<td>100% (15/15)</td>
</tr>
<tr>
<td>Frattini 2008²</td>
<td>Total DNA, <em>KRAS, p16INK4a</em></td>
<td>Surgery ± chemotherapy</td>
<td>70</td>
<td>100% (18/18)</td>
</tr>
<tr>
<td>Ryan 2003³</td>
<td><em>KRAS</em></td>
<td>Surgery ± chemotherapy (53%)</td>
<td>94</td>
<td>91% (10/11)</td>
</tr>
<tr>
<td>Tie 2016⁴</td>
<td>Personalised assay</td>
<td>Surgery ± chemotherapy</td>
<td>230</td>
<td>41% (11/27) in surgery-only group</td>
</tr>
</tbody>
</table>

* (n positive ctDNA / n recurrence)

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MONITORING TUMOR BURDEN AND RESPONSE ON TREATMENT

• 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)

### ctDNA AND TREATMENT OUTCOMES

<table>
<thead>
<tr>
<th>Reference</th>
<th>Gene(s)</th>
<th>Treatment</th>
<th>Patients (N)</th>
<th>Detection rate in plasma, %*</th>
<th>Predictive value</th>
<th>Predictive value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lefebure 2010¹</td>
<td>KRAS, RASSF2A methylation</td>
<td>Chemotherapy</td>
<td>31</td>
<td>52% (12/23)</td>
<td>Shorter PFS in patients with positive ctDNA (median 5 vs 14 months; ( P=0.004 ))</td>
<td></td>
</tr>
<tr>
<td>Spindler 2014²</td>
<td>Total cfDNA and KRAS</td>
<td>Cetuximab + irinotecan</td>
<td>73</td>
<td>–</td>
<td>Higher response rate in patients with &gt;50% decrease in total cfDNA at cycle 3 (40% vs 17%; ( P=0.04 ))†</td>
<td>Poor disease control in patients with high levels of KRAS-mutant ctDNA (0% vs 42%; ( P=0.048 ))‡</td>
</tr>
<tr>
<td>Spindler 2012³</td>
<td>Total cfDNA and KRAS</td>
<td>Cetuximab + irinotecan</td>
<td>108</td>
<td>78% (32/41)</td>
<td>Low baseline cfDNA associated with higher disease control rate</td>
<td></td>
</tr>
</tbody>
</table>

* (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.

cfDNA LEVEL HAS A STRONG PROGNOSTIC VALUE IN CRC

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

TRACKING RESISTANCE AND TAILORING THERAPY

• 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 \textit{RAS} mutations for acquired resistance to anti-EGFR therapy\textsuperscript{1-4}.
  • Numerous secondary \textit{KRAS} mutations have been identified in serum ctDNA and may be detected 5-10 months before radiologic documentation of disease progression\textsuperscript{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,\textsuperscript{5} and \textit{MET} amplification\textsuperscript{6}.

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

CONCLUSION

• 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