Detection of variable methylation patterns improves sensitivity of a colorectal cancer blood test

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BACKGROUND
We have previously evaluated the accuracy of a novel DNA blood test across a spectrum of benign and neoplastic conditions in the colon/rectum using a multiplexed methylation-specific PCR assay for detection of circulating fully-methylated BCA1 and IKZF1 DNA (Assay 1). However, a number of methylation patterns are likely to exist in a colorectal neoplasm due to the heterogeneous nature of human solid tumours.

AIM
To determine if a modified 2-gene blood test that enables detection of partial methylation of three interprimer CpG sites in the IKZF1 target region (Assay 2) improves detection of cancer.

MATERIALS AND METHODS
Cell-free DNA recovered from 4mL plasma from colonoscopy confirmed subjects was bisulphite-converted and assayed for the presence of methylated BCA1 and IKZF1 DNA (Pedersen et al., 2015). The BCA1 MethyLight assay was multiplexed with one of two different IKZF1 MethyLight assays (Assay 1 or Assay 2; see Assay Details below). The IKZF1 PCR component in Assay 1 constituted three methylation-specific oligonucleotides (forward and reverse primers, plus a hydrolysis probe) for amplification and detection of a fully methylated 95-bp target region. The IKZF1 PCR component in Assay 2 used the same two methylation-specific IKZF1 primers as Assay 1, but used a ‘degenerate’ hydrolysis probe cocktail, synthesised with a 50:50 mix of C and T at three interprimer CpG sites. Thus, Assay 2 would detect eight different interprimer methylation patterns in the 95-bp IKZF1 target region.

RESULT 1: Variable methylation at IKZF1

IKZF1 amplicons were sequenced from bisulphite-converted DNA extracted from colonoscopy-confirmed cancer cases that were positive (left panel) or negative (centre and right panel) with the fully-meth IKZF1 probe.

• The centre and right panels give examples of cancers that show variable methylation at the IKZF1 locus.
• The IKZF1 assay was thus redesigned to include a mixture of 8 probes to detect all possible methylation combinations (See IKZF1 Probe (Assay 2), Assay details).

RESULT 2: ASSAY 2 POsITIVITY

<table>
<thead>
<tr>
<th>Clinical status</th>
<th>N</th>
<th>Full-meth n</th>
<th>Vari-meth n</th>
<th>McNemar P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-neoplastic</td>
<td>514</td>
<td>8</td>
<td>1.6</td>
<td>17</td>
</tr>
<tr>
<td>Adenomas</td>
<td>196</td>
<td>3</td>
<td>1.5</td>
<td>11</td>
</tr>
<tr>
<td>TIs (stage 0)</td>
<td>2</td>
<td>0</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>Cancers</td>
<td>33</td>
<td>11</td>
<td>33.3</td>
<td>17</td>
</tr>
</tbody>
</table>

• A subset of 743 previously assayed samples (See panel at left) were re-assayed with Assay 2, allowing detection of variably-methylated IKZF1.
• IKZF1 positivity increased for all cancers from 33.3% to 51.5% (p=0.04), and particularly for early stage (I+II) cancers from 25% to 46% (p=0.07).

RESULT 3: ASSAY 2 CLINICAL PERFORMANCE

<table>
<thead>
<tr>
<th>Genomic Positivity (%)</th>
<th>2013: Full-meth assay n = 677 (467N, 175A, 2 Stage 0, 33 CRC)</th>
<th>2014: Partial IKZF1 meth assay n =</th>
<th>Difference</th>
</tr>
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<tbody>
<tr>
<td>Non-neoplastic</td>
<td>64%</td>
<td>70%</td>
<td>6%</td>
</tr>
<tr>
<td>Adenomas</td>
<td>67%</td>
<td>81%</td>
<td>14%</td>
</tr>
<tr>
<td>Stage I+II</td>
<td>56%</td>
<td>67%</td>
<td>11%</td>
</tr>
<tr>
<td>Stage III+IV</td>
<td>17%</td>
<td>22%</td>
<td>5%</td>
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</table>

• When combined with BCA1 results, Assay 2 resulted in an increase from 64% to 70% sensitivity for cancers, with a 92% specificity.
• The increased sensitivity was due to an increase in positivity in early stage cancers.

CONCLUSIONS
• Allowing detection of variably-methylated IKZF1 improved blood-test sensitivity for early stage cancers.
• IKZF1 methylation may be incompletely in early cancers.
• Seeking opportunities to further investigate clinical utility.

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ASSAY 1 POSITIVITY
Assay 1 positivity rate by clinical status

<table>
<thead>
<tr>
<th>Clinical status</th>
<th>n</th>
<th>2010</th>
<th>CVP 2-gene panel</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-neoplastic</td>
<td>1283</td>
<td>74</td>
<td>5.8</td>
</tr>
<tr>
<td>Non-advanced Adenomas</td>
<td>460</td>
<td>30</td>
<td>6.5</td>
</tr>
<tr>
<td>Advanced Adenomas</td>
<td>232</td>
<td>17</td>
<td>7.3</td>
</tr>
<tr>
<td>TIs (stage 0)</td>
<td>3</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>Cancers</td>
<td>130</td>
<td>85</td>
<td>65.4</td>
</tr>
</tbody>
</table>

Stage I 24 7 29.2
Stage II 53 36 67.9
Stage III 39 30 76.9
Stage IV 9 8 88.9
Unstaged 5 4 80.0

65% sensitivity (any cancer) / 94% specificity

Young et al., DDW 2014

ASSAY DETAILS

• BCA1 Fwd primer: 5’-GGTTTTTTTGTTTTTTGGTTAGTTG
• BCA1 Rev primer: 5’-CAAGCCGCAAAGACCIACAC
• BCA1 Probe: HEX-5’TTCTGCGGCAGGGCTGTT-3’FAM
• IKZF1 Fwd primer: 5’-GACGACGTATTTTTTTCGTGTTTC
• IKZF1 Rev primer: 5’-CCCCGCGGCTTCTGGACCG
• IKZF1 Probe (Assay 1): FAM-5’-TTGTGACGATGGATGCGGAG-BHQ1
• IKZF1 Probe (Assay 2): FAM-5’-TTGTGATGGATGCGGAG-BHQ1

• ACTB Fwd primer: 5’-TTTGATCTTCTGGATTCT
• ACTB Rev primer: 5’-GGCGCAATCTCTGGACCG
• ACTB Probe: TaqMan FAM-5’-AGGCGTGTTAGTGACGCAGC

PCR conditions:
• 30 cycles, 1 min extension at 72°C;  15 min initiation at 95°C
• 95°C for 15 sec; 62°C for 40 sec

• When combined with BCA1 results, Assay 2 resulted in an increase from 64% to 70% sensitivity for cancers, with a 92% specificity.
• The increased sensitivity was due to an increase in positivity in early stage cancers.

REFERENCES

• Young, GP; Pedersen, SK; Dekker, E; Cole, SR; Osborne, JM; Symonds, EL; Mallon-Hent, RC; Moovey, A; Baker, R; Gaur, S; Murray, DH, Lapointe, LC. Evaluation of a 2-gene (IKZF1 and BCA1) DNA blood test for detection of colorectal cancer. Digestive Disease Week, Chicago 2014. Gastroenterology 2014;79(5) Suppl AB128.