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

Rohan T. Baker, Snigdha Gaur, David Murray, Aidan McEvoy, Graeme Young*, Lawrence LaPointe, and Susanne K. Pedersen
Clinical Genomics Pty Ltd, North Ryde, NSW, Australia. *Flinders Centre for Innovation in Cancer, Adelaide, SA, Australia

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 *BCAT1* 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 *BCAT1* and *IKZF1* DNA (Pedersen et al., 2015). The *BCAT1* 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.

Assay Details

- BCAT1 Fwd primer: 5'-GTTTTTTTTGTTGATGTAATTCGTTAGGTC
- BCAT1 Rev primer: 5'- CAATACCCGAAACGACGACG
- BCAT1 Probe: HEX-5'-TTCGTCGCGAGAGGGTCGGTT-IbFQ
- IKZF1 Fwd primer: 5'-GACGACGTATTTTTTTCGTGTTTC
- IKZF1 Rev primer: 5'-GCGCACCTCTCGACCG
- IKZF1 Probe (Assay 1): FAM-5'-TTTGTATCGGAGTAGCGATTCGGGAG-IbFQ
- IKZF1 Probe (Assay 2): FAM-5'-TTTGTATYGGAGTAGYGATTYGGGAGG-IbFQ (Y = 50% C, 50% T).
- ACTB Fwd primer: 5'-GGAGTTTTTGTTTTTTGGTTAGTTG
- ACTB Rev primer: 5'-CAAAATAAAATACAAAACAAACCTAATCC
- ACTB Probe: Tex615-5'-ATGGAGGTTTAGTGGTAATATAGGTTTTGTTTGG- IbRQ
- PCR conditions:
  - 30µL final PCR volume; 12µL bisulfite-converted DNA; 15µL 2X Quantitect NOROX (QIAGEN); 3µL 10X oligo mix.
  - All primers at 200nM final; all probes at 100nM final
  - 1 cycle, 15min/95°C; 50 cycles: 15sec/95°C; 40sec/62°C with acquisition (FAM, HEX and Texas Red).
  - Roche LC480 II with colour-compensation applied.

ASSAY 1 POSITIVITY

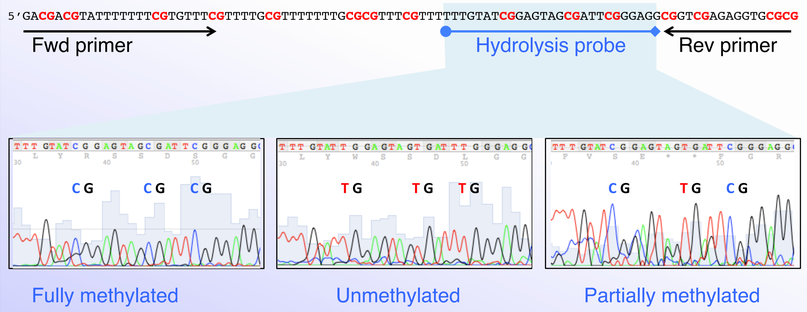
Assay 1 positivity rate by clinical status

Clinical status	n 2108	CVP 2-gene panel n	CVP 2-gene panel %
Non-neoplastic	1283	**74**	**5.8**
Non-advanced Adenomas	460	30	6.5
Advanced Adenomas	232	**17**	**7.3**
TIs (stage 0)	3	0	-
Cancers	130	**85**	**65.4**
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

RESULT 1: Variable methylation at *IKZF1*



- *IKZF1* amplicons were sequenced from bisulphite-converted cfDNA 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

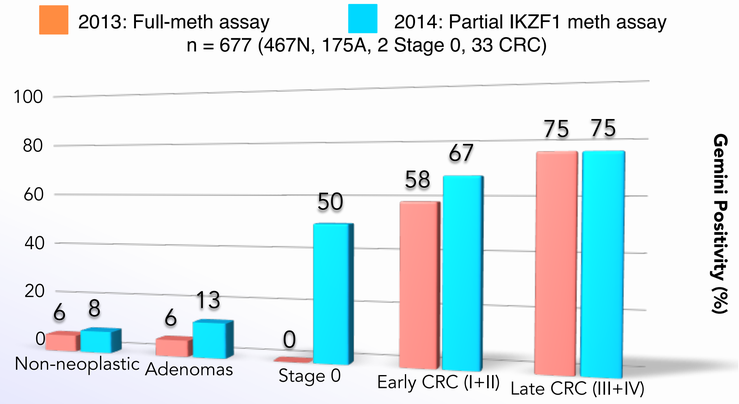
Assay 2: *IKZF1* positivity rate

Clinical status	N 743	Full-meth n	Full-meth %	Vari-meth n	Vari-meth %	McNemar P
Non-neoplastic	514	8	1.6	17	3.3	0.08
Adenomas	196	3	1.5	11	5.6	0.04
TIs (stage 0)	2	0	-	1	50	N/A
Cancers	33	11	33.3	17	51.5	0.04
Stage I+II	24	6	25	11	45.8	0.07
Stage III+IV	9	5	55.6	6	66.7	1.00

- 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

Assay 2 in a clinical cohort



64% sensitivity/94% specificity → 70% / 92%

- When combined with *BCAT1* 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

- Pedersen SK, Baker RT, McEvoy A, Murray DH, Thomas M, Molloy PL, Mitchell S, Lockett T, Young GP, LaPointe LC. A Two-Gene Blood Test for Methylated DNA Sensitive for Colorectal Cancer. *PLoS One*. 2015 Apr 30;10(4):e0125041. doi: 10.1371/journal.pone.0125041.
- Young, GP, Pedersen, SK, Dekker, E, Cole, SR, Osborne, JM, Symonds, EL, Mallant-Hent, RC, Mcevoy, A, Baker, R, Gaur, S, Murray, DH, Lapointe, LC. Evaluation of a 2-gene (*IKZF1* and *BCAT1*) DNA blood test for detection of colorectal cancer. Digestive Disease Week, Chicago 2014. Gastroenterology 2014;79(5) Suppl AB125

CONCLUSIONS

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

info@clinicalgenomics.com