CLINICAL EVALUATION OF A NOVEL DUPLEXED 2-GENE DNA METHYLATION BLOOD TEST FOR COLORECTAL CANCER

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BACKGROUND

A blood test to screen for colorectal neoplasia may improve participation rates and may be more specific for neoplasia-associated molecular changes than FIT. We have described discovery of genes down-regulated in colorectal carcinomas and adenomas compared to normal tissue, and subsequent bisulfite deep sequencing to guide development of methylation-specific PCR (MSP) assays for initial validation in tissue and then plasma samples[1]. Two genes previously not known to be methylated in colorectal cancer, IKZF1 (Ikaros family zinc-finger 1) and BCAT1 (branched-chain aminotransferase 1), were evaluated as separate MSP assays in a colonoscopy-confirmed cohort of 251 patient plasma specimens (including 74 cancers) and demonstrated a high sensitivity for cancers (IKZF1: 68%; BCAT1: 65%; combined: 76%) and little/no methylated DNA in normal plasma (either gene: 3%, combined: 7%)[1].

AIM

To develop, optimise and test a BCAT1/IKZF1 duplexed MSP assay, to minimise plasma volume required for clinical testing.

MATERIALS and METHODS

- In previous work, BCAT1 was probe-based (Hex) and IKZF1 was Sybr Green)[1]. A Fam probe was thus designed for the existing IKZF1 amplicon (Table I).
- The duplex assay was optimised for mastermix, primer and $MgCl_2$ concentration and thermal cycling parameters on a standard curve of bisulphite-converted (Epitect Plus, Qiagen) fully methylated human genomic DNA (CpGenome, Millipore) in a background of bisulphite-converted human genomic DNA (Roche), with 5ng total DNA per well, containing from 5ng to 20pg bis-methylated DNA.
- Circulating DNA was extracted from 4mL plasma from colonoscopy-confirmed patients using QiaAmp Circulating Nucleic Acid kit on the QiaCube (Qiagen) with 40uL elution and manual re-elution.
- Bisulphite-conversion and DNA purification used Epitect Fast bisulphite kits and QiaCube (Qiagen) with 40uL elution and manual re-elution. Final eluate volume 36uL.
- Assays were performed in triplicate using 10uL eluate (equivalent to ~1mL plasma) per replicate.
- The duplexed assay was tested on an unblinded cohort of 33 cancer/33 normal plasma samples against each singleplex assay, followed by a blinded cohort of 499 plasma samples drawn from patients just prior to undergoing colonoscopy.

RESULTS

Optimised duplex assay (Table I) showed the same LoD as either singleplex assay (Figure 1)

- Assayed on a standard curve of bisulphite-converted fully methylated DNA in a background of bisulphite-converted unmethylated DNA

The duplex assay performs similarly to combined singleplex assays in the unblinded clinical sample test set

- In the unblinded sample set, the duplexed assay was positive in 3/33 normals and 18/33 cancers, similar to the combined performance of the two singleplexed assays, 1/33 normals and 19/33 cancers. These differences are not statistically significant.

The duplex assay performed similarly to previous data in a large, blinded cohort (Figure 2; Table II)

- In all clinical samples, patient positivity was defined as any PCR signal from any of the 3 wells for either gene.
- In the blinded 499-patient cohort, the duplexed assay was positive in 23/326 normals (7%), 9/154 adenomas (6%), and 12/19 cancers (63%). Of the cancers, the assay detected 2/7 (29%) Stage I, 5/6 (83%) Stage II, and 5/6 (83%) Stage III; no Stage IV cancers were studied. Full patient breakdown is given in **Table II.** Patient positivity did not correlate with total DNA input (measured by ACTBbis qPCR assay), patient age, or gender (data not shown).
- The duplexed assay thus gave similar specificity and sensitivity to previous data from combined singleplex assays (see Background).

CONCLUSIONS and FUTURE DIRECTIONS

- Hypermethylation of gene loci IKZF1 and BCAT1 is observed in DNA extracted from blood plasma of most cancer patients, but few normals or patients with adenomas. The reason(s) for non-detection of adenomas is unclear but might include their lack of vascularisation.
- Assaying for both genes is additive and improves the detection rate for cancers.
- The duplexed-MSP assay described here provides the same sensitivity and specificity as our previous separate singleplexed assays, and thus reduces the required volume of plasma for the same diagnostic outcome.
- The duplexed assay is now being tested in expanded clinical trials relative to established screening methods including colonoscopy and FIT.

TABLE I: PCR Conditions

Probe-based assay details

BCAT1/IKZF1 Duplex
BCAT1 Forward: 5' GTTTTTTTGTTGATGTAATTCGTTAGGTC
BCAT1 Reverse: 5' CAATACCCGAAACGACGACG
BCAT1 Probe: 5' HEX - TTCGTCGCGAGAGGGTCGGTT - BHQ1

IKZF1 Forward: 5' GACGACGTATTTTTTTCGTGTTTC
IKZF1 Reverse: 5' GCGCACCTCTCGACCG
IKZF1 Probe: 5' FAM - TTTGTATCGGAGTAGCGATTCGGGAG - BHQ1

Assay conditions: GoTaq Hot Start mastermix (Promega; 1X) with 4mM (total) MgCl2, 200nM each primer, 100nM each probe. 10uL template into 30uL final PCR volume.
Cycling conditions (LC480 II): 1 cycle 95°C/2 min; 50 cycles 95°C/15sec, 62°C/30sec, 72°C/30sec with acquisition.

Conversion-specific QC assay details

ACTBbis singleplex
ACTB Forward: 5' GTGATGGAGGAGGTTTAGTAAGTT
ACTB Reverse: 5' AATTACAAAAACCACAACCTAATAAA
ACTB Probe: 5' FAM - ACCACCACCCAACACACAATAACAAACACA - BHQ1

Assay conditions: Platinum Taq (Invitrogen; 0.05U/uL) with 3mM MgCl2, 200uM dNTPs, 900nM primers and 100nM Probe. 5uL template into 15uL final PCR volume.
Cycling conditions (LC480 II): 1 cycle 95°C/2 min; 50 cycles 95°C/10sec, 60°C/50sec with acquisition

Figure 1: Standard curves; duplex vs singleplex

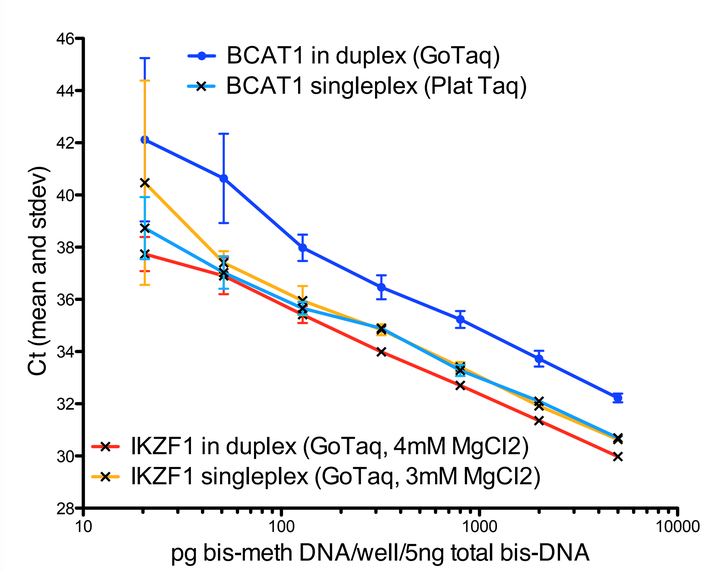

Figure 2: Duplex assay on 499 blinded samples

93% Specificity, 63% Sensitivity (any cancer)

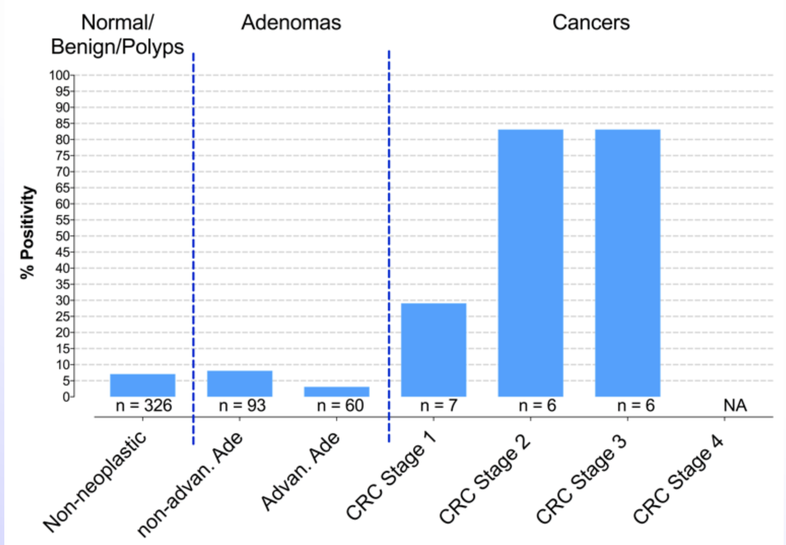

TABLE II: Patient breakdown

499 specimens	n	% of total	$\bar{x}$ age (yr)	♀/♂	BCAT1[d] n +ve, %	IKZF1[d] n +ve, %	GEMINI n +ve, %
Non-Neoplastic[a]	326	65%	61	168/158	23, 7%,	5, 2%	23[b], 7%
Adenoma	154	31%	65	71/83	8, 5%	3, 2%	9, 6%
Non-advanced[c]	93	19%	64	42/51	6, 6%	3, 3%	7, 8%
Advanced	61	12%	66	29/32	2, 3%	0	2, 3%
Cancer	19	4%	73	11/8	10, 53%	6, 32%	12, 63%
I	7	1%	71	4/3	2, 29%	0	2, 29%
II	6	1%	77	3/3	5, 83%	2, 33%	5, 83%
III	6	1%	70	4/2	3, 50%	4, 67%	5, 83%
IV	0	0%	-	-	-	-	-

[a] Includes colonoscopy confirmed healthy controls (n=119) and benign polyps (n=207) such as hemorrhoids, diverticulosis, angiodysplasia, IBD and hyperplastic polyps. [b] 23 of 326 non-neoplastic samples were BCAT1 positive of which only 3 of the 23 where IKZF1 positive. The 23 positive non-neoplastic specimens included 9 healthy patients (39%) whereas the remaining patients (61%) had benign polyps such as diverticulosis, haemorrhoids and angiodysplaia [c] Non-advanced adenoma: Tubular and < n=3, and <10mm, and low-grade dysplasia; otherwise adenomas were "Advanced". [d] A positive 'score' was assigned to any specimen with at least one positive methylation signal.

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Reference:
1. Baker, R.T. et al., (2012) J. Gastroenterol. Hepatol. 27 (Suppl. 4), 123-124.