Development and clinical performance of an accurate cell-free DNA (cfDNA) methylation assay for early detection of colorectal cancer

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BACKGROUND

- Colorectal cancer (CRC) is the 3rd most commonly diagnosed cancer worldwide, and its incidence is further increasing in a younger population (1-2)
- Established CRC screening methods include fecal immunochemical testing, which requires handling of stool, and colonoscopy, which is invasive. Many people who remain unscreened are believed to accept a blood-based screening test, which could have a profound public health impact (3)
- DNA methylation is a stable, early and tissue-specific event in cancer development and progression (4)
- Measuring the methylation status of tumor-derived cell-free DNA in plasma could enable identification of early-stage CRC
- Here, we demonstrate the development and validation of a plasma-based methylated DNA panel for all stages of CRC with emphasis on early stages

METHODS

- In the initial phase of potential marker selection, we analyzed the colon cancer Human Methylation 450K data available from The Cancer Genome Atlas (TCGA) consortium (5)

Patients:

- In Study 1 (Training Set), candidate regions were evaluated and predictions models generated in plasma samples of 215 patients (Table 1)
- In Study 2 (Validation Set), the selected markers were validated in an independent multi-centric validation set of 774 samples that included samples from lung and breast cancer (Table2). 20 additional samples from Study 1 were used for quality control purposes

Analytical method:

- Methylation-sensitive restriction enzyme (MSRE) –qPCR approach was used to design assays for regions of interest
- 1/3 of plasma extracted cfDNA was directly pre-amplified and analyzed by µ-fluidic qPCR
- 2/3 of extracted cfDNA was digested with methylation sensitive restriction enzymes-AciI, Hin6I or HpyCH4IV (1-15 cut sites per target) and analyzed by µ-fluidic qPCR

Statistical analysis and prediction model

- The most promising methylation markers in plasma were selected by analyzing Study 1 samples
  - Random forest feature selection algorithm utilizing Monte-Carlo cross-validation over 50 sub-setting used
- Reduced list of 53 significant hypermethylated marker assays ($p<0.01$) in plasma of colorectal cancer patients compared to healthy control and non-advanced adenoma (NAA) patients was analyzed on Study 2 samples
- Quality control was performed analyzing reproducibility of the marker values on 20 replicate samples
- Reproducible markers were used for building SVM-based prediction algorithm on Study 1 samples
- 12-marker SVM algorithm was then applied on Study 2 samples for validation purpose

Table 1 Study 1 (Training Set) sample distribution

	CRC	Control + NAA	Control	NAA
Female	46	59	46	13
Male	47	63	45	18
Total	**93**	**122**	**91**	**31**
Total sample	**215**			

	CRC All	Proximal	Distal
Localized	54	23	31
Advanced	39	22	17
Total	**93**	**45**	**48**

Table 2 Study 2 (Validation Set) sample distribution

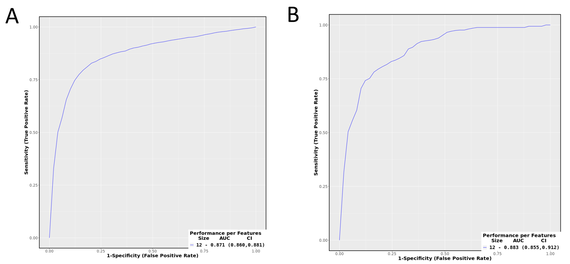
	CRC	Control + NAA	Control	NAA	Breast C.	Lung C.
Female	71	296	223	73	26	6
Male	81	274	199	75	0	20
Age (range)	65 (41-84)		61 (41-82)	66 (50-82)	55 (41-77)	61 (48-75)
Total	**152**	**570**	**422**	**148**	**26**	**26**
Total samples	**774**					

	CRC All	Breast C.	Lung C.
Stage 0-I	35	16	15
Stage II	49	8	4
Stage III	48	2	6
Stage IV	13		
Unknown	7		1
Total	**152**	**26**	**26**

RESULTS

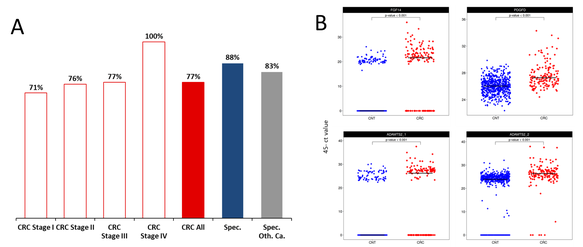
- Publicly available tissue data analysis yielded 180 potential regions of interest
- 53 markers significantly hypermethylated on plasma samples
- 12- marker SVM prediction algorithm was built on Study 1 samples using reproducible markers leading to initial Training Set prediction performance accuracy of AUC 87% (CI: 86-88%) (Figure 1 A)
- 12-marker prediction algorithm further validated on large independent Study 2 sample set of 774 that included samples from colorectal cancer, NAA, control and also lung and breast cancer patients to test the marker-panel tissue-specificity towards colorectal cancer
- Model performance on Study 2 validation set can be seen on Figure 1 B with AUC 88% (CI: 85.5-91%) and overall sensitivity of 77% and specificity of 88% (Figure 2 A)

Figure 1 Performance of 12-marker panel on validation set *A. ROC curve and AUC for Study 1 (Training Set). B. A. ROC curve and AUC for Study 2 (Validation Set) that includes samples of colorectal cancer, non-advanced adenoma, controls and patients with lung and breast cancers*



- Individual methylation marker analysis showed a clear hypermethylation in colorectal cancer samples compared to other conditions. Individual p-values for all markers $p< 0.01$ (Figure 2 B)

Figure 2 Detection of methylated markers in plasma. *A. Accuracy values, where red bar represents the overall sensitivity for CRC, red-outlined bars represent sensitivity by stage and blue bar represents specificity (healthy + non-advanced adenoma+ other cancer) and grey bar represents specificity comparing CRC to other cancers. B. 45 – Ct values plotted for 4 markers for control (healthy+ non-advanced adenoma+ other cancers) samples (blue) and CRC samples (red). Higher 45 – Ct values correspond to higher methylation status*



CONCLUSIONS

- We developed a colorectal cancer prediction model based on a novel plasma 12-marker methylation panel
- We demonstrated high accuracy of the developed model for early-stage CRC detection in a 774 sample validation study
- We showed that selected markers are tissue specific towards colorectal cancer
- We concluded that this method could serve as the basis for a highly accurate and minimally invasive blood-based CRC screening test

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