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Detection of Methylated DNA in Plasma from Colorectal Cancer Patients and Controls by Real-Time PCR Analysis of Septin 9

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Abstract

Background – Colorectal cancer is one of the most common cancers world-wide and patients have been shown to benefit from early detection of the disease through regular screening. New population-based screening tests should attain both high sensitivity and high specificity to be effective. Tests should also be easily available and acceptable to the patient. Our approach is to provide an easily administered, blood-based test for detection of colorectal cancer.

Methods – Plasma samples were collected from patients with colorectal cancer and polyps to test sensitivity of the methylation-based marker assay and from colonoscopy-verified healthy individuals to test specificity. In addition, to identify clinical conditions that could result in false positives, we analyzed a number of critical controls including various cancers other than colorectal cancer and acute and systemic conditions. For the analysis of samples, free-DNA was first extracted from plasma samples using an automated MagNaPure device, then bisulfite treated to preserve methylation information and lastly analyzed for methylation of the Septin 9 gene by real-time PCR.

Results – Real-time PCR analysis of a single methylation-based biomarker in free-DNA from colorectal cancers plasmas resulted in detection of 176/336 (52.4%). In the colonoscopy verified healthy samples 15/316 were detected (4.7%). 4/45 polyps < 1 cm were detected (8.8%) and 15/65 polyps > 1cm were detected (23.1%). Samples from patients with other serious medical conditions were also analyzed although patients with these diseases would not normally be undergoing a screening test. Of 93 other cancer plasma tested, 9 were found to be positive (9.7%) and of 196 acute local and systemic diseases, 23 were detected (11.7%).

Conclusion – Methylation analysis of free-DNA from plasma using a real-time assay for the Septin 9 gene results in sensitive and specific detection of colorectal cancer. This blood-based test has promise for improved patient compliance because of ease of use including lack of dietary restrictions and omission of bowel preparation. Improvement by addition of markers complementary to Septin 9 will make this test an attractive alternative to other screening methods.

Note – An additional methylation marker, ALX4, was found in a subsequent independent study to have high sensitivity for colorectal adenomas. Paneled with the Septin 9 marker, sensitivity for adenomas, particularly large adenomas with dysplasia is greatly increased.

Colorectal Cancer Screening Test – Current Process Workflow

Table 1 – Septin 9 Study

Group	# Positives/Total Tested	% [95% CI]
CRC*	**176/336**	**52 [47,58]**
CRC-Stage I	27/62	44 [31,57]
CRC-Stage II	33/80	41 [30,53]
CRC-Stage III	65/111	59 [49,68]
CRC-Stage IV	41/62	66 [53,76]
Polyps < 1cm	4/45	9 [2,21]
>= 1cm	15/65	23 [14,35]
Healthy Controls	**15/316**	**5 [3,8]**
Non-colorectal diseases	**23/196**	**12 [8,17]**
Non-colorectal cancers	**9/93**	**10 [5,18]**

* Includes 1 stage 0 CRC sample and 20 CRC samples of unknown stage.

Table 3-Septin 9+ALX4 Panel by polyp histology

Group	# Positives/ Total Tested	% [95% CI]
Tubular or villous adenoma (all sizes)	20/36	56 [38,72]
Tubular or villous adenoma + > 1cm	11/11	100 [72,100]
Adenoma with IEN* (all sizes)	8/10	80 [44,97]
Hyperplastic polyps	4/13	31 [9,61]

* IEN-intraepithelial neoplasia

Table 2 – Septin 9+ALX4 Panel Study

Assay	Group	# Positives/ Total Tested	% [95% CI]
Septin 9	**CRC**	2/5	40 [5,85]
	Polyps < 1cm	3/31	10 [2,26]
	>= 1cm	3/18	17 [4,41]
	Healthy Controls	1/22	5 [0,23]
ALX4	**CRC**	2/5	40 [5,85]
	Polyps < 1cm	13/31	42 [25,61]
	>= 1cm	10/18	56 [31,78]
	Healthy Controls	**4/22**	**18 [5,40]**
Septin 9 + ALX4	**CRC**	3/5	60 [15,95]
	Polyps < 1cm	12/31	39 [22,58]
	>= 1cm	12/18	67 [41,87]
	Healthy Controls	**2/22**	**9 [1,29]**

Materials and Methods

Study population – Samples were collected from male and female patients ages 40 to 80 but predominantly ages 50 and older. Four main categories of samples were collected from patients with all stages of CRC, polyps, cancers other than CRC, non-cancerous diseases and from age-matched colonoscopy-verified healthy controls. Case report forms were reviewed by a physician and severity of disease determined. Samples from patients with late stage (symptomatic) cancer in the non-colorectal cancer group or patients with severe disease in the non-cancerous disease category were excluded from this analysis.

Plasma methylation analysis – Plasma was processed first by extraction of free-circulating DNA using the Total Nucleic Acid DNA extraction kit (Roche Applied Science) and Roche MagNaPure device. Eluted DNA's from each patient were pooled and concentrated on microcon filters. DNA Methylation information was preserved by deamination of unmethylated cytosines using sodium bisulfite. Bisulfite treated plasma DNA in each sample was quantified on the Roche LC 2.0™ device using a non-methylation specific assay for b-actin. Septin 9 methylation was determined using an assay based on Epigenomics' HM real-time PCR technology.[1] The 90% limit of detection of the Septin 9 assay was estimated as 21pg by a dilution series of methylated (SSS1 treated) DNA in a background of 50ng blood DNA (Roche human genomic DNA). The Roche LC 2.0 was also used to measure Septin 9 amplification. A plasma equivalent of 1.6 ml to 1.9 ml of DNA was added per PCR reaction and each plasma sample run in duplicate or triplicate.

Quality control and statistical analysis – Positive and negative control samples were run in each process step to determine fluctuations per process batch. Based on a process calibration phase, the MagNaPure extraction and bisulfite treatment steps were calibrated and batches in which control DNA concentrations were outside the range of 3 standard deviations were excluded from analysis. Sample numbers were pre-determined to provide acceptable confidence intervals. A sample was considered positive if at least 2 of the replicates were positive. Amplification curves were analyzed automatically and also by two independent reviewers to validate true curves. Discrepancies were resolved by a third, independent reviewer.

Septin 9 – The Septin 9 gene, also called MSF, encodes a mammalian septin protein that when disrupted results in incomplete cell division.[2] Septin 9 and other proteins have been shown to be fusion partners of the proto-oncogene MLL suggesting a role in tumorogenesis.[3] Septin 9 has also been shown in loss of heterozygosity (LOH) studies to be in a frequently deleted region in breast and ovarian cancers, a finding that further implicates the gene as a possible tumor suppressor.[4]

Septin 9 + ALX4 Panel for improved polyp sensitivity – In a previous study we identified ALX4 as a methylation marker with high sensitivity for polyps.[5] ALX4 is a putative transcription factor that belongs to the family of paired-class homeoproteins. We collected an independent set of plasma samples and measured the amount of methylated DNA in plasma for both markers as described above. A sample was considered positive if at least 3 of the 6 (Septin 9 or ALX4) replicates were positive.

Conclusions

In this report we determine the sensitivity of a real-time PCR assay for methylated Septin 9 DNA. Sensitivity for detection of colorectal cancer of all stages was 52%. Sensitivity for detection of polyps larger than 1cm was 23%. Our results indicate that the Septin 9 biomarker is also highly specific (95%) in asymptomatic individuals over 50 years of age. Specificity was also high (88–90%) in a population of patients with clinical conditions such as gastritis, arthritis, respiratory infection and early stage cancers other than CRC. The marker was shown to detect colorectal cancer with similar sensitivity regardless of stage of progression or location of the lesion in the colon unlike fecal tests such as FOBT, and iFOBT that have been shown to have a decreased sensitivity for both proximal colorectal cancers and early stage cancers.[6]

Early results of combining Septin 9 and ALX4 in a marker panel indicate that sensitivity for detecting polyps can be considerably improved while maintaining a high specificity. The panel of both methylation markers detected polyps larger than 1cm with a sensitivity of 67%. Sensitivity for large adenomas (> 1cm) or adenomas with IEN showed even greater improvement (100%, 80% respectively). Specificity of the panel in asymptomatic individuals over 50 years of age was 91%.

Patient compliance and performance of current screening strategies limit the effectiveness of tests available on the market today. An easily administered blood-based test for early detection of colorectal cancer followed by colonoscopy for positive individuals has the potential to be a very effective tool for reducing mortality from this disease.

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