Potential of a New Blood Test for Colorectal Cancer Screening – The Septin 9 Gene Biomarker

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Abstract

Despite clear evidence for a better prognosis when detected early, in most countries colorectal cancer (CRC) has a low compliance rate in terms of screening. There are several methods of CRC screening ranging from a variety of stool tests, e.g. faecal occult blood test (FOBT), to endoscopy (sigmoidoscopy, colonoscopy). A blood test for CRC detection is a new alternative, at least for patients not willing to accept screening colonoscopy or to undergo FOBT. The septin 9 biomarker is a potential candidate to fulfil this purpose. It has been validated in several case–control studies, showing a strong association of plasma-containing methylated DNA within the septin 9 gene (^{m}SEPT9) with the presence of CRC. If sensitive methylated-DNA-detection technologies are used for ^{m}SEPT9 detection in blood plasma samples, sensitivities of about 50% for stage I, 70–80% for stages II and III and 90–100% for stage IV at a specificity of ≥90% have been reported in these studies. Screening experts assume that such a blood-based test will increase compliance to CRC screening. Further studies are ongoing or have just been completed, including a large prospective screening trial involving 8,000 individuals in the US and Germany. The main objectives of this clinical investigation, called Prospective Evaluation of Septin 9 Performance for Colorectal Cancer Screening (PRESEPT), are to determine the performance of the septin 9 test for identification of CRC in a screening population and to demonstrate the health economic benefit of septin 9 in this setting. Results are expected in April 2010. This article presents an update on current analytical and clinical data on ^{m}SEPT9.

Colorectal Cancer

Colorectal cancer (CRC) is among the most common cancers in the developed world. With the average lifetime risk of developing CRC as high as 6%, CRC represents a major health burden in these societies. In the US, approximately 150,000 people were diagnosed with the disease in 2008 and about 50,000 people died of this cancer in the same period.[1] In Europe, 413,000 people were diagnosed and 207,000 people died of the disease in 2006.[2] However, CRC is among the most curable cancers when detected early, with a five-year survival rate of 90%. The survival rate drops dramatically to about 10% with metastatising stage IV cancers.[3] The tumorigenesis of CRC is relatively well understood on both a morphological and a genetic level. Therefore, the cancer is thought to develop over a time-frame of about 10 years, mostly via pre-malignant lesions such as colorectal adenomas. Over the course of disease progression, epigenetic and genetic alterations accumulate in the pre-neoplastic and neoplastic epithelial cells of the colon and rectum, increasingly compromising growth control and local organ confinement of the cancer.[4]

Colorectal Cancer Screening – Guidelines and Adherence in Western Countries
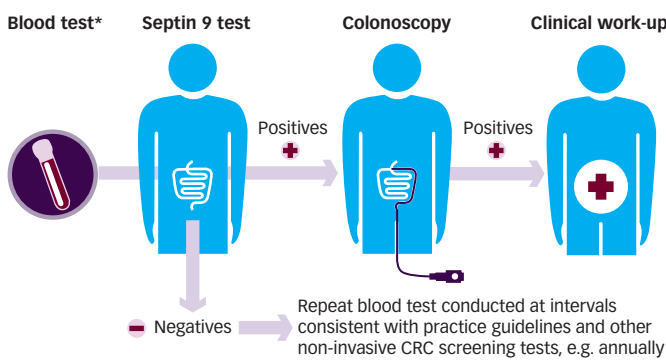
High prevalence and curability at low costs in the early stages but high mortality and treatment costs with advanced disease make a good case for widespread CRC screening. Indeed, it has been shown that population-based screening can reduce mortality from CRC. Four independent studies in the US and Europe have demonstrated that even screening with guaiac-based faecal occult blood tests (FOBTs), which have a comparatively low sensitivity, could reduce mortality by 13–21% (biannual testing) or 33% (annual testing) when one-third to half of the eligible population complied with screening recommendations.[5] Consequently, cancer-screening guidelines in most countries in the industrialised world recommend CRC screening by non-invasive and/or invasive diagnostic procedures, most commonly FOBT and/or – less frequently – colonoscopy. For example, German guidelines by the Joint Committee of Physicians and Health Insurers in October 2002 recommend annual FOBT testing starting from 50 years of age followed by two colonoscopies after a 10-year interval starting from 55 years of age, or biannual FOBT testing. Both FOBT and colonoscopy are reimbursed under the public healthcare scheme. Despite an intensive public debate, Switzerland does not have a national screening programme for CRC. However, a number of private and public initiatives advertise CRC screening by colonoscopy or FOBTs starting from 50 years of age. CRC screening in general is not reimbursed in Switzerland. French healthcare authorities promote FOBT testing by repeated personal invitation of eligible individuals. However, both colonoscopy and FOBTs are recommended for

Table 1: Clinical Performance of Septin 9 Tests in Plasma Specimen

Study	Samples	Sensitivity	Specificity	Workflow
1[18]	312	52	95	Experimental workflow
2[18]	600	57	96	
3[18]	725	48	93	
4[19]	370	48	96	
5[19]	550	72	90	
6[20]	269	73	93	*m*SEPT9-detection Assay
7[20]	245	69	89	
Performance Evaluation[23]	257	67	88	Epi *pro*Colon™ Early Detection Assay
Total	3,328			

Figure 1: Principle of Colorectal Cancer Screening Utilising the Septin 9 Test

Blood test*   **Septin 9 test**   **Colonoscopy**   **Clinical work-up**

Positives +

Positives +

− Negatives

Repeat blood test conducted at intervals consistent with practice guidelines and other non-invasive CRC screening tests, e.g. annually

*A simple blood test is part of the annual physical examination to indicate presence of colorectal cancer (CRC) and to direct patients to colonoscopy.

screening and reimbursed by the national healthcare system. Similar to France, the National Health Service (NHS) in the UK aggressively promotes the use of standard FOBTs for screening by personal invitation, free supply of test kits to the homes of patients and other measures. However, colonoscopy is not reimbursed for primary screening, but used as a second-line screening modality after a positive FOBT.[6] Guidelines in the US recommend annual testing by FOBT and colonoscopies at 10-year intervals, with flexible sigmoidoscopies half-way between the colonoscopies. Screening with guideline-recommended methods is generally reimbursed provided that the patients have access to some kind of healthcare scheme.[7]

Despite these recommendations and a multitude of measures to enforce them by healthcare providers, healthcare authorities and patient advocacy groups, the majority of CRCs continue to be diagnosed in advanced stages.[8,9] Adherence to screening programmes remains a noticeable challenge: in Germany, ~3% of the target group per year accept a screening colonoscopy. Extrapolated to the recommended 10-year interval, more than 70% of the population between 55 and 65 years of age have never undergone a screening colonoscopy.[10] In the US, about 40% comply with recommendations to undergo screening by endoscopy in the recommended time interval.[11] Non-invasive screening modalities would be potentially suitable to better motivate screened individuals to undergo a colonoscopy if they were accepted by the screening population. However, compliance to FOBT is estimated to be as low as 20% in Germany and 12% in the US.[10,11] One reason for the low adherence specifically to FOBT screening may be the level of patient involvement. The patient is required to undergo the inconvenience of taking several stool samples at home; in addition, dietary restrictions have to be adhered to with the use of conventional guaiac FOBTs. Alternative stool-based methods, including immunochemical FOBTs (iFOBTs), M2-PK and single-strand DNA (sDNA) testing, despite fewer restrictions on their use, did not fundamentally change the picture, although some of the immunological stool tests may perform better.[12] In fact, it could also be that the wide variety of available stool-based tests that superficially appear to be very similar, not only to patients but also to primary care physicians (PCPs), may lead to confusion rather than to increased compliance.

There are a variety of tests in terms of CRC screening, with colonoscopy being the most accurate, but it also has the highest risk and a variable, mostly low compliance. Screening can only be effective if the majority of the target population complies with the screening recommendations. A blood-based test for CRC screening is an interesting alternative. It can be integrated into a regular check-up including physical exam and other blood parameters, does not involve an additional invasive procedure other than phlebotomy and does not have any risks. Therefore, compliance is likely to be increased compared with invasive screening methods such as colonoscopy, but this remains to be shown in demonstrated studies.

Blood-based Colorectal Cancer Screening with the Septin 9 Test
DNA Methylation of the Septin 9 Gene

In tumorigenesis, epigenetic alterations are among the early events that occur in cells changing from normal to tumour cells. Methylation of DNA, the addition of a methyl group to the 5' position of cytosine, defines one type of epigenetic change that is frequently related to a change at the expression level of a gene. This has been well characterised in tumour biology in general,[12,13] and extensively described for CRC in particular.[14–16]

Epigenomics has identified and validated DNA methylation of the septin 9 gene (*SEPT9*) as a potential biomarker for CRC. The *SEPT9* codes for the septin 9 protein, a member of a conserved family of guanosine triphosphate (GTP)-binding proteins. Septins are multifunctional proteins involved in vesicle trafficking, apoptosis, cytoskeletal remodelling, infection, neurodegeneration and neoplasia (i.e. cancer), among other important cellular processes. The septin 9 methylation biomarker (*m*SEPT9) was discovered in tissue through genome-wide screening. Cytosine residues in the v2 region of *SEPT9* become methylated in CRC tissue, but not in normal colon mucosa.[17] This tumour-specific methylation pattern can be used to amplify specifically cell-free DNA shed into the bloodstream by tumour cells. Since the development of a sensitive realtime polymerase chain reaction (PCR) assay, *m*SEPT9 performance has been verified in plasma specimens in multiple independent case–control studies (see *Table 1*).[18–20]

In order to make *m*SEPT9 testing available to the general population, Epigenomics has developed a CE-marked *in vitro* diagnostic (IVD) test for reliable detection of *m*SEPT9 in a blood plasma specimen according to respective regulations for IVD products. This test reports the presence of *m*SEPT9 in a qualitative way for easy interpretation. As the presence of *m*SEPT9 is strongly associated with the detection of invasive colorectal adenocarcinoma, a patient testing positive for *m*SEPT9 should undergo colonoscopy to confirm the diagnosis (see *Figure 1*). For patients who have a negative septin 9 test result, the

Figure 2: Assay Procedure Epi *pro*Colon™
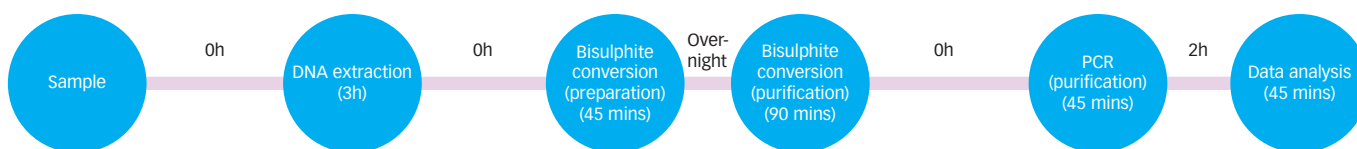
PCR = polymerase chain reaction. Lag times between the hands-on steps (black text); hands-on time (white text).

physician and patient can jointly decide what future screening is necessary, for example re-testing at regular intervals. Such approaches have to be validated in future prospective trials.

Technological Principles of the Methylated Septin 9 Detection Test

The principle of detection of methylated septin 9 DNA consists of four steps (see *Figure 2*), as detailed below.

Blood Sampling and Plasma Preparation

A 9–10ml blood sample is taken in the physician's office using pre-defined K_2EDTA blood collection tubes and kept at 2–8ºC until collection by the laboratory courier, and is then transported refrigerated to the central laboratory. On delivery to the laboratory, but within 24 hours after the blood is taken, the blood plasma is separated by repeated centrifugation and stored at 2–8ºC for further processing.

DNA Extraction from Plasma

At the laboratory, cell-free DNA of about 3.5ml of plasma is captured, washed and concentrated using magnetic beads that are covered with a suitable DNA-binding matrix. In broad terms, binding buffer containing the magnetic beads is added to the plasma and incubated under agitation. The beads are captured using a magnet and the supernatant is discarded. The DNA attached to the beads is washed several times with a washing buffer and then eluted from the beads in a small volume of elution buffer. All reagents necessary for this step are provided by the Epi *pro*Colon™ Plasma DNA Preparation Kit.

Bisulphite Conversion and DNA Purification

By treating the DNA with a high concentration of bisulphite under denaturing conditions, unmethylated cytosin is converted into uracil, while methylated cytosin is unaffected. Thus, methylation patterns are converted into sequence information that can be interrogated by any technology that discriminates between sequences, including realtime PCR. The conversion is achieved by combining the DNA with the bisulphite reagent and a reaction buffer for an overnight incubation. After the incubation, the DNA is purified by a second DNA extraction using magnetic particles with a similar protocol to that used in the first extraction from blood plasma.

DNA Methylation Biomarker Detection via Realtime Polymerase Chain Reaction

An *in vitro* PCR assay for the qualitative detection of *SEPT9* gene methylation uses Epigenomics' proprietary realtime PCR technologies HeavyMethyl and MethyLight.[21] The assay detects the previously methylated bisulphite-converted DNA of the targeted region in the sample. Thus, the previously methylated DNA is amplified in the presence of blockers that prevent amplification of previously unmethylated DNA. As a second level of discrimination, realtime PCR

Table 2: Results of the Analytical Performance Evaluation

	0pg/ml Methylated DNA	8pg/ml Methylated DNA	16pg/ml Methylated DNA
Valid replicates	24	23	24
*m*SEPT9-positive	0	23	23
*m*SEPT9-negative	24	0	1

specifically probes for previously methylated DNA, which is used for PCR fragment detection. The *m*SEPT9 biomarker assay is multiplexed with the actin-beta assay used as an internal control to assess the validity of the result. Furthermore, positive and negative workflow controls are processed with each test run. All reagents required for steps two to four are provided by the Epi *pro*Colon product line, comprising kits for plasma DNA preparation, realtime PCR and full workflow positive and negative controls, all available from Epigenomics.

Performance Characteristics of the Methylated Septin 9 Detection Test
Analytical Performance

To determine the limit of detection, a panel of three technical samples was tested. Two different concentrations (8 and 16pg/ml) of hydrolysed DNA detectable with the *m*SEPT9 PCR were spiked into a matrix containing genomic DNA at a concentration of 10ng/ml. A blank sample consisting of matrix with genomic DNA only and the two technical samples were processed with 24 replicates each, by three technicians. Bisulphite-converted DNA was prepared using reagents from different lots of the Epi *pro*Colon Plasma DNA Preparation kit, and the converted DNA was assayed with Epi *pro*Colon realtime PCR kits from different lots adopting a schedule from NCCLS EP 17-A.[22] All 24 replicates of the blank sample were determined *m*SEPT9-negative and 23 replicates of the technical samples containing 8 and 16pg/ml DNA spikes were determined *m*SEPT9-positive. *Table 2* summarises the data obtained.[23] Therefore, the detection limit of the assay is a concentration equivalent to as few as three genome copies per millilitre.

Reproducibility

The reproducibility of the procedure was tested by processing plasma aliquots from four pools generated from human ethylene diamine tetraacetic acid (EDTA) plasma. Two of the plasma pools comprised plasma from patients diagnosed with invasive colorectal adeno-carcinoma. Two pools comprised plasma from patients with no apparent disease as controls. Both cancer pools were processed in nine replicates and both non-cancer pools were processed in 18 replicates. Processing of replicates was performed by three operators using different lots of the Epi *pro*Colon Plasma DNA Preparation kit (M5-01-001) and the Epi *pro*Colon realtime PCR kit. In 51 of the 54 *m*SEPT9 determinations, the expected result (cancer pool *m*SEPT9-positive; non-cancer pool *m*SEPT9-negative) was consistently generated.[23]

Clinical Performance

The clinical performance of the Epi *pro*Colon realtime PCR kit was tested in a case–control design comprising specimens from histologically confirmed invasive colorectal adenocarcinoma patients of all stages and specimens from colonoscopy-verified negative individuals with no apparent disease. Bisulphite-converted DNA was prepared using the Epi *pro*Colon Plasma DNA Preparation kit from 261 plasma aliquots from individual subjects and converted DNA was assayed with the Epi *pro*Colon realtime PCR kit. Based on internal and external control values for each run, valid *m*SEPT9 measurements were determined for 257 out of 261 specimens (98.5%). Of the 154 subjects with no evidence of disease (controls), 135 specimens were determined *m*SEPT9-negative, resulting in an estimated clinical specificity of 88% (95% confidence interval [CI] 82–92%). Of the 103 subjects diagnosed with invasive colorectal adenocarcinoma (cases), 69 specimens were determined *m*SEPT9-positive, resulting in an estimated clinical sensitivity of 67% (95% CI 57–76%). *m*SEPT9 was positive in 44 of the 66 patients with cancer in early, still localised disease states (44 stage I, 22 stage II). *Table 2* summarises the results.

PRESEPT

Prospective Evaluation of Septin 9 Performance for Colorectal Cancer Screening (PRESEPT) is a multicentre clinical study sponsored by Epigenomics to prospectively evaluate the clinical performance of *m*SEPT9 for colorectal cancer screening, as defined by the US Multisociety Task Force on Colorectal Cancer. This study provided data within the framework of a real screening setting with a presumably low prevalence of CRC (estimated 50 cancers from about 7,500 screening cases), in contrast to many other tests with data only from case–control studies. Clinical data obtained under International Conference on Harmonisation/World Health Organization Good Clinical Practice (ICH/GCP) control focus mainly on CRC detection as the main outcome measure, but will also provide data on polyp detection such as advanced adenomas and others as secondary study aims. Health economic analyses will be performed to assess the possible economic benefit. The study was closed in December 2009 with 7,914 individuals enrolled and 51 cancers detected, and the first preliminary results are to be expected in April 2010 after plasma has been tested at three testing centres, two in the US and one in Germany. Results of the PRESEPT study will provide objective evidence of the performance of *m*SEPT9-based CRC screening.

Conclusions and Outlook

The newly developed *m*SEPT9 early detection assay (Epi *pro*Colon) is the first CE-marked IVD assay for early detection of CRC in a blood plasma specimen. The design and robustness of the IVD assay enables its use in standard routine laboratory procedures. The convenience of the test for the patient and the PCP has the potential to improve patient compliance with CRC screening. The Epi *pro*Colon test now makes it possible to screen patients for CRC who might not otherwise be detected due to their denial of other screening modalities. What role this test will play in the concert of screening measures has to be determined after the results of the PRESEPT trial and further studies on compliance and cost–benefit. ■

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