Evaluation of a novel liquid biopsy-based ColoScape assay for mutational analysis of colorectal neoplasia and triage of FIT+ patients: a pilot study

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ABSTRACT

Circulating cell free tumour derived nucleic acids are becoming recognised as clinically significant and extremely useful biomarkers for detection of cancer and for monitoring the progression of targeted drug therapy and immunotherapy. Screening programmes for colorectal cancer in Europe use the Fetal Immunochemical Test (FIT) test as a primary screener. FIT+ patients are referred to immediate colonoscopy and the positive predictive value (PPV) is usually 25%. In this article, we report a study employing the ColoScape assay panel to detect mutations in the APC, KRAS, BRAF and CTNNB1 genes, in order to collect preliminary performance indicators and plan a future, larger population study. The assay was evaluated on 52 prospectively collected whole-blood samples obtained from FIT+ patients enrolled in the CRC screening programme of ASL NAPOLI 3 SUD, using colonoscopy as confirmation. The assay's sensitivity for advanced adenomas was 53.8% and the specificity was 92.3%. The PPV was 70.0% and negative predicitive value (NPV) was 85.7%. Workflow optimisation is essential to maximise sensitivity. Of note, four of the six positive cases missed by ColoScape had a less than suboptimal DNA input (data not shown). Had they been ruled out as inadequate, sensitivity would have increased from 53.8% to 69%. However, as stated previously, this is not a clinical trial, but rather an initial, preliminary technical evaluation. In conclusion this study shows that ColoScape is a promising tool and further studies are warranted in order to validate its use for the triage of FIT+ patients.

BACKGROUND

Most cancers are caused by acquired mutations in the body's somatic cells. Investigating these mutations and their role in triggering the progression from benign to malignant lesions advances our understanding of tumour evolution which should lead to earlier detection and prevention.[1] Analysis of somatic mutations by measuring the circulating tumour DNA (ctDNA) component in peripheral blood ('liquid biopsy') can assist with non-invasive screening, treatment and monitoring of cancer management.[2–4] ctDNA is a component of cell-free DNA (cfDNA) that can be detected and used as an indicator for the presence of a tumour.[2–5]

Varying types of tumours have been shown to produce high levels of cfDNA in plasma,[6–9] and several studies have identified mutations and quantified ctDNA in subjects with advanced stages of cancer or patients undergoing treatment. As the stage of disease increases, the prevalence of ctDNA also increases. Early detection of colorectal cancer is served by currently available clinically approved tests such as the Fetal Immunochemical Test (FIT), which detects the presence of blood in stool and the centralised stool DNA based test (Exact Sciences Cologuard) which also includes the FIT test in its methodology, which requires a method to isolate the minute amounts of human (tumour derived) DNA from a vast excess of microbial nucleic acids found in human stool samples. Since patients' plasma is a convenient source of pathological nucleic acids, this circulating cfDNA can be readily isolated from a blood draw which is performed on a regular basis by all physicians and the mutational landscape of this can be interrogated on a dynamic basis.

Colorectal cancer is the third most frequent cancer in the world. Approximately 1.7 million new cases were diagnosed in 2015, with about 832 000 deaths. The progression from precancer to cancer and metastasis is relatively slow, averaging 15 years. This creates an opportunity for early detection and successful treatment. In Europe, the test of choice in most screening programmes is the faecal immunochemical test for the detection of blood in the stool (FIT).[10] Patients who test positive at FIT are referred to colonoscopy, where, however, about 75% of them turn out to be negative.[11] An intermediate test with good sensitivity and specificity could help select FIT+ patients at greater risk to be positive at colonoscopy. Researchers all over the world have focused their attention on mutational analysis with a view to identifying biomarkers that could aid in the early detection of colo-rectal cancer (CRC) and/or its recurrences. Some important results have been obtained in late stage and metastatic cancer, where mutational analysis is now routinely used prior to prescribing some novel biological therapies.[12] The assessment of wild-type status in the RAS gene is a prerequisite to the use of cetuximab and panitumumab, to give an example.[13] On the other hand, not much experience and literature exist on molecular analysis in early detection of CRC. An

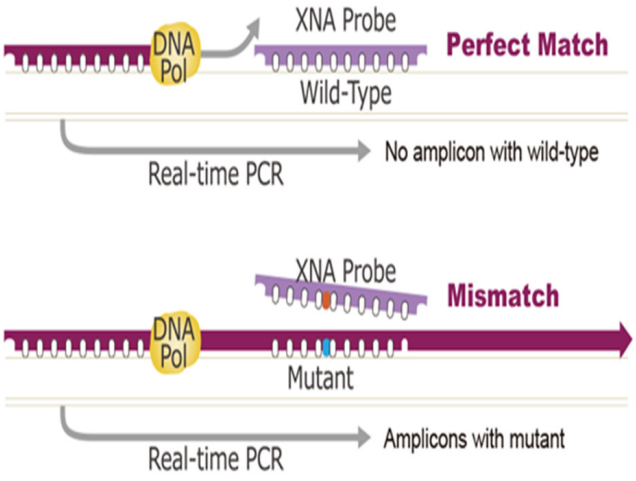

Figure 1 Principle of the QClamp ColoScape mutation test in targeted genes. XNA, xeno nucleic acid.

article published by Imperiale *et al* in the NEJM in 2014[14] described an FDA-approved stool-DNA test (Cologuard, Exact Sciences, Madison, Wisconsin, USA) and reported sensitivity of 42% for advanced adenomas and 92% for cancer, with a specificity of 87%. Other work has been done employing Septin 9 (Epigenomics), another FDA-approved test based on detection of methylation markers in blood samples.[15] DiaCarta Inc., a molecular diagnostic based in Richmond (California), has developed a highly sensitive multiplex real-time qPCR assay that combines a multiplex colorectal gene specific panel[7 8] with proprietary xeno nucleic acid (XNA) wild-type clamping probe technology. XNA allows the selective DNA polymerase amplification of only target nucleic acid templates that contain mutations, while blocking wild-type templates, thus maximising analytical sensitivity.

In this preliminary pilot study, the sensitivity and specificity of this multiplex qPCR assay for the detection of tumour specific somatic mutations in patients circulating cfDNA were investigated in order to collect some initial performance parameters as a basis to design a follow-on study of adequate power and sample size that will provide information for the assay's potential use in the triage of FIT+ patients. This is not a clinical trial. It is a pilot study and no inferences should be made from it.

MATERIALS AND METHODS

Patient and sample collection

Sixty patients referred to colonoscopy for a FIT+ test were enrolled by the Gastroenterology Department of ASL Napoli 3 Sud – Hospital S. Maresca of Torre del Greco. Informed consents were obtained and 5–20 mL of blood were drawn from each patient and stored in cfDNA BCT Streck tubes.

Plasma separation and DNA extraction

Whole-blood samples were transferred to the processing laboratory (Predictive Molecular Pathology Laboratory, Department of Public Health, University Federico II of Naples), where the plasma was separated using the previously described double-spin.[16] Approximately 2–10 mL of plasma were obtained from each sample and frozen for later use. cfDNA was extracted using QIAamp MiniElute cfDNA Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Evaluation of DNA quality and quantity was performed on TapeStation 4200 (Agilent, Santa Clara, California, USA).

Table 1 ColoScape cycling parameters on ABI QuantStudio 5

Step	Temperature (°C)	Time (s)	Ramp rate (°C/s)	Cycles	Data collection
Preincubation	95	300	1.6	1	OFF
Denaturation	95	20	1.6	X50	OFF
XNA annealing	70	40	1.6		OFF
Primer annealing	66	30	1		OFF
Extension	72	30	1		FAM and VIC

ColoScape assay test

The ColoScape kit (DiaCarta, Richmond, California, USA) is a real-time PCR based in vitro diagnostic assay for the detection of colorectal cancer associated mutations in genes including APC (codons 1309, 1367, 1450,) KRAS (codons 12 and 13), BRAF (codon 600) and CTNNB1 (codons 41 and 45) in plasma and formalin-fixed paraffin-embedded (FFPE).[17] The assay can be performed on DNA extracted from either FFPE or plasma samples to identify the presence or absence of mutations in the targeted regions but does not specify the exact nature of the mutation. The QClamp technology used by the ColoScape assay is based on XNA mediated PCR clamping technology. XNA is a synthetic DNA analogue in which the phosphodiester backbone has been replaced by a novel synthetic backbone chemistry. XNAs hybridise tightly to complementary DNA target sequences only if the sequence is a complete match. Binding of XNA to its target sequence blocks strand elongation by the DNA polymerase. When there is a mutation in the target site, and therefore a mismatch, the XNA-DNA duplex is unstable, allowing strand elongation by the DNA-polymerase. Addition of an XNA, whose sequence is a complete match to the wild-type DNA, to a PCR reaction, blocks amplification of wild-type DNA allowing selective amplification of mutant DNA.[17] XNA oligomers are not recognised by DNA-polymerases and cannot be used as primers in subsequent real-time PCR reactions (figure 1). The test was performed on ABI QuantStudio 5 instrument according to DiaCarta's protocol and the cycling parameters are presented in table 1.

RESULTS

Limit of detection

To determine the limit of detection (LoD) and analytical sensitivity of the assay, studies were performed using serial dilutions of genetically defined genomic DNA reference standards from cell lines with defined mutations obtained from Horizon Discovery (Cambridge, England) and cfDNA reference standards from SeraCare (Massachusetts, USA). Mutant allelic frequencies tested were 1%, 0.5% and 0.1% at 2.5 and 5 ng DNA input. Each sample was tested in 20 replicates and 95% correct calling was used as the criteria for determination of LoD (tables 2 and 3). At 5 ng cfDNA input, all targets could be detected at 1% mutant allelic frequency and 0.5% mutant allelic frequency could be detected for APC 1309, APC 1450 and CTNNB1 41 targets.

In addition, precision studies involving intra-assay and inter-assay reproducibility, lot to lot variation and operator variability showed good reproducibility with CV% below 4%.

Table 2 LoD results determined using cfDNA reference standards

Target mutation	DNA input, ng/well	5	2.5
		% correct call	% correct call
APC 1309	1% mutation	100	83
	0.5% mutation	100	83
APC 1367	1% mutation	100	82
	0.5% mutation	92	75
APC 1450	1% mutation	100	100
	0.5% mutation	100	100
CTNNB1 41	1% mutation	100	100
	0.5% mutation	100	60
CTNNB1 45	1% mutation	100	90
	0.5% mutation	83	60
KRAS 12	1% mutation	100	79
	0.5% mutation	79	29
KRAS 13	1% mutation	100	70
	0.5% mutation	83	67
BRAF V600	1% mutation	100	100
	0.5% mutation	85	79

cfDNA, cell-free DNA; LoD, limit of detection.

Cell-free DNA extraction and quality control

cfDNA was successfully extracted from all patient plasma samples and no genomic DNA contamination was observed based on TapeStation analysis (data not shown). The estimated cfDNA concentrations varied ranging from 0.4 to 9.0 ng/µL and, as expected, the extracted cfDNA concentrations from 10 mL plasma were higher than those from 2 to 5 mL (median 2.9 vs 1.6 ng/µL).

ColoScape assay test and colonoscopy result comparison

Of the total 60 patient samples, there were 52 valid samples. Eight samples were excluded from analysis due to either a missing colonoscopy report or technical reasons. Advanced precancerous lesions included all advanced adenomas and sessile serrated polyps measuring 1 cm or more in size. No cancers were found in this sample set. Colonoscopy was used as the truth throughout to calculate performance indicators, using international recommended guidelines to identify advanced adenomas. Out of 52 valid samples, 13 showed positive colonoscopy results among which 7 were tested positive by ColoScape assay with a sensitivity being 53.8%. Among 39 samples with negative colonoscopy results, 36 samples were tested as negative by ColoScape assay with a specificity being 92.3% (tables 4 and 5). The results of 10 samples tested positive by ColoScape assay are presented in table 6.

Table 3 LoD results determined using genomic DNA reference standards

Target mutation	DNA input, ng/well	5	2.5	
		% correct call	% correct call	
	1% mutation	100	100	
APC 1309	0.5% mutation	100	90	
	1% mutation	100	90	
APC 1367	0.5% mutation	100	20	
	1% mutation	100	100	
APC 1450	0.5% mutation	100	95	
	1% mutation	100	100	
CTNNB1 41	0.5% mutation	100	100	
	1% mutation	100	100	
CTNNB1 45	0.5% mutation	100	95	
	1% mutation	100	100	
KRAS 12	0.5% mutation	100	67	
	1% mutation	100	60	
KRAS 13	0.5% mutation	80	50	
	1% mutation	100	100	90
BRAF V600	0.5% mutation	100	75	70

LoD, limit of detection.

Table 4 Summary of colonoscopy and ColoScape results

	Colonoscopy positive	Colonoscopy negative	Total
ColoScape positive	7	3	10
ColoScape negative	6	36	42
Total	13	39	52

DISCUSSION

cfDNA is a challenging type of sample for mutational analysis. Estimates for ctDNA range from 1% to 10% of cfDNA. In addition, mutations can occur at different allelic frequencies, which may be in some cases as low as 0.1%.[11] XNA aims to maximise analytical sensitivity due to its ability to selectively amplify only, or predominantly, mutant forms and block wild-types. The manufacturer recommends a minimum of 5 ng of DNA per reaction, although there is evidence that that assay works with a 2.5 ng DNA input. The aim of this pilot study was to assess the performance of ColoScape in detecting advanced adenomas from blood samples in addition to establishing a robust clinical and laboratory workflow. It is important to note that four of the six positive cases missed by ColoScape had a less than suboptimal DNA input (data not shown). Had they been ruled out, sensitivity would have increased from 53.8% to 69%. However, as stated previously, this is not a clinical trial, but rather an initial, preliminary technical evaluation. The most prevalent mutation was found in the KRAS gene (four cases). Other mutations were APC (two cases) and CTNNB1 (one case) and BRAF in one case of dual positivity with KRAS. Interestingly, a case (#28) of a polyp with size of 4 mm, which did not meet the positivity criteria, showed a KRAS positivity and Sanger sequencing confirmed the presence of a KRAS c.35G>A; p.G12D mutation. One case that was excluded due to inadequate bowel preparation was negative and showed no relevant genetic variations.

CONCLUSION

Given the small sample size, sensitivity, specificity and resulting predictive values, only estimates that will help design and power a future clinical trial must be considered. However, it is of considerable interest to consider that detection of advanced

Table 5 Key performance indicators for ColoScape assay

ColoScape assay	Proportion	%	95% CI
Detection rate of AA	7/52	13.5	(4.2 to 22.7)
Sensitivity for AA	7/13	53.8	(26.7 to 80.9)
Specificity	36/39	92.3	(83.9 to 100.0)
Positive predictive value	7/10	70.0	(41.6 to 98.4)
Negative predictive value	36/42	85.7	(75.1 to 96.3)

AA, advanced precancerous lesions.

Table 6 Results of 10 samples tested positive by ColoScape assay

Sample ID	Colonoscopy results	ColoScape results
18	Positive	KRAS 12 positive
19	Positive	APC 1450 positive
24	Positive	KRAS 12 positive
28	Negative (1 polyp of 4 mm not meeting positivity criteria)	KRAS 12 strong positive (KRAS c.35G>A; p.G12D confirmed by Sanger sequencing)
35	Negative	APC 1450 positive
40	Negative	KRAS 12 positive
45	Positive	KRAS 12 and BRAF 600 positive
50	Positive	CTNNB1 45 positive
54	Positive	APC 1450 positive
55	Positive	KRAS 12 positive

adenomas is a real challenge for screening programmes that are based on the FIT test, and for the other clinically approved molecular tests, such as Cologuard and Septin 9. One has to also consider specificity that should ideally exceed 90% in order to rule out a significant number of FIT+ patients that now turn out negative on colonoscopy. This pilot study justifies further investigation of the ColoScape assay as a non-invasive tool to detect cancers and advanced adenomas in a triage setting. The most important result obtained from this study was the identification of a clinically relevant workflow that can optimise performance and allows us to estimate the sensitivity and specificity of the assay that will be the main focus of the future trial. Other interesting aspects to be investigated will be: management of FIT+, triage —patients, management of FIT+, triage+ and colonoscopy—patients, management of patients with inadequate bowel preparation. Based on the results from this study, further studies are warranted in order to validate the use of liquid biopsy-based ColoScape assay for the triage of FIT+ patients.

Take home messages

- The detection of advanced adenomas is a real challenge for screening programmes that are based on the FIT test and other molecular tests.
- This pilot study justifies further investigation of the ColoScape assay as a non-invasive tool to detect cancers and advanced adenomas in a triage setting.
- In this study we identify a clinically relevant workflow to estimate the sensitivity and specificity of the ColoScape assay in the future trial.

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Competing interests F-PS and MS are consultants of DiaCarta Inc. JD, QS and MJP are employees of DiaCarta Inc. Other authors have no competing interests to declare.

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