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Highly sensitive, non-invasive detection of colorectal cancer mutations using single molecule, third generation sequencing

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ABSTRACT

Colorectal cancer (CRC) represents one of the most prevalent and lethal malignant neoplasms and every individual of age 50 and above should undergo regular CRC screening. Currently, the most effective preventive screening procedure to detect adenomatous polyps, the precursors to CRC, is colonoscopy. Since every colorectal cancer starts as a polyp, detecting all polyps and removing them is crucial. By exactly doing that, colonoscopy reduces CRC incidence by 80%, however it is an invasive procedure that might have unpleasant and, in rare occasions, dangerous side effects. Despite numerous efforts over the past two decades, a non-invasive screening method for the general population with detection rates for adenomas and CRC similar to that of colonoscopy has not yet been established. Recent advances in next generation sequencing technologies have yet to be successfully applied to this problem, because the detection of rare mutations has been hindered by the systematic biases due to sequencing context and the base calling quality of NGS.

We present the first study that applies the high read accuracy and depth of single molecule, real time, circular consensus sequencing (SMRT-CCS) to the detection of mutations in stool DNA in order to provide a non-invasive, sensitive and accurate test for CRC. In stool DNA isolated from patients diagnosed with adenocarcinoma, we are able to detect mutations at frequencies below 0.5% with no false positives. This approach establishes a foundation for a non-invasive, highly sensitive assay to screen the population for CRC and the early stage adenomas that lead to CRC.

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1. Introduction

Although the rate of colorectal cancer (CRC) has been declining at 3.0% per year over the past decade, in 2014 there were about 140,000 new cases in the United States making it the third most common cancer after lung-bronchus and prostate (Cancer Facts and Figures, American Cancer Society, 2014) and this figure in North America essentially mirrors the latest available worldwide survey (www.globocan.iarc.fr).

A substantial amount of research has been conducted in the past thirty years to demonstrate that the molecular genetic landscape of CRC is extremely complex. Single Nucleotide Polymorphisms (SNPs), Insertion–Deletions (Indels), Microsatellite Instabilities (MSI), and alteration in methylation patterns can all occur at different loci depending on the site of tumor and its stage (Fearon, 2011; The Cancer Genome Atlas Network, 2012; Tomlinson et al., 2010; Houlston, 2012).

The molecular mechanisms behind the formation of adenomas and their progression into CRC were first presented 25 years ago in a model proposed by Fearon and Vogelstein (Fearon and Vogelstein, 1990).

Briefly, early adenomas emerge on the normal epithelium and this event is associated with mutations in the Adenomatous Polyposis Coli (*APC*) or β-catenin (*CTNNB1*) genes (Morin et al., 1997; Sparks et al., 1998). Most early adenomas suffer additional mutations in either the Kirsten Rat Sarcoma (*KRAS*) or v-raf murine sarcoma viral oncogene homolog B (*BRAF*) genes (or other genes of the RTK-RAS pathway), which lead to the formation of intermediate and larger adenomas (Chan, 2003). It is at this point that chromosomal instabilities (CIN) or deficiency in the mismatch repair (MMR) system begin to occur, which leads to an increased mutation rate in the neoplastic cells. CIN in colorectal cancer is often observed on the long arm of chromosome 18 and it is associated with mutations in genes *SMAD2* and *SMAD4* (Takagi et al., 1996; Miyaki et al., 1999). These genes represent human homologs of mothers

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against decapentaplegic (*MAD*) in drosophila and of the SMA protein in *Caenorhabditis elegans*. On the other hand, MMR is usually caused by a decrease in the expression of the MutL-homolog 1 (*MLH1*) gene on chromosome 3 through the hyper methylation of the promoter region (Vilar and Gruber, 2010). MMR induces alteration of microsatellite sequences in the tumor cells and increases the overall mutation rate for all genes, including oncogenes and tumor suppressor genes (Parsons et al., 1993). Finally, the progression to carcinoma is often accompanied by the malfunctioning of the cellular tumor antigen *TP53*, a protein with tumor suppressor activity.

The progression of CRC shows a significant acceleration between the second and the third stage, i.e., upon the onset of CIN or MMR, with survival rates dropping from 80% to less than 40%, whereas early detection (stage I) is associated with survival rates above 90% (Sameer, 2013). It is therefore critical to base the screening of the general population on approaches capable of confidently identifying the first, local instances of adenomas and CRC neoplastic formation.

The methods currently used to test for CRC can be divided into two general categories: invasive methods such as colonoscopy, and non-invasive methods that detect biomarkers in stool. Colonoscopy, i.e., the direct inspection of the colon, is certainly the most accurate approach as the physician is able to visualize the epithelium and eventually ascertain the presence of abnormalities on its surface. However it presents a number of drawbacks. It can still miss a significant percentage of adenomas, even upon repeated examination and it tends to have unpleasant, and in rare occasions, dangerous side effects, which are mostly connected to the preparation that the bowel requires prior to the procedure (Senore et al., 2011; Lebwohl et al., 2011). Moreover, this preparation represents an additional step in this screening process which seems can be sub-optimally performed in a significant number of cases (Lebwohl et al., 2011). Not the least, its invasiveness is the source of reluctance for a considerable number of individuals, particularly among certain populations (Ramos et al., 2011; Talaat and Harb, 2013). Despite its lower level of discomfort, since only the rectum and the lower colon are inspected, similar arguments can be put forward for flexible sigmoidoscopy (FSG) and computed tomography colonography (Senore et al., 2011).

Non-invasive tests based on fecal occult blood (FOBTs) are a widely used screening method. A small sample of stool is self-collected, placed on a card, and sent to the physician. There are several methods for testing for occult blood in the feces: for instance, fecal immunochemical testing (FIT) utilizes antibodies to detect human hemoglobin whereas in guaiac tests (gFOBT) the stool is smeared on a chemically treated paper which, if blood is present, will change color when absorbing hydrogen peroxide. FIT has been shown to perform more effectively than gFOBT (Sharp et al., 2012), however FOBTs in general are much less effective in reducing CRC incidence compared to colonoscopy (Moayyedi and Achkar, 2006), mostly due to their inherent difficulties in detecting early stages polyps, which do not bleed.

Finally, non-invasive testing of stool can be done by interrogating the genetic material present in a stool sample. The use of DNA testing for screening is certainly extremely promising, however so far it has been hindered by its limited sensitivity since, when the presence of mutated DNA is to be inferred from body fluids, the concentration of mutated cells becomes extremely low. Being continuously available and easy to collect and containing mutant DNA from exfoliating adenomas at the earliest stages of CRC development, stool is the obvious choice for inspecting potential CRC mutations and it is not surprising that results based on stool samples are superior to those based on cell free DNA in blood plasma (Diehl et al., 2008). Early studies using multi-target panels, based on methylation, mutation and hemoglobin assays reported sensitivities between 70% and 90% for CRC, where the sensitivity would typically increase with the number of markers included and the size of the neoplasm (Dong et al., 2001; Ahlquist et al., 2000; Ahlquist et al., 2012; Traverso et al., 2002a). Recently, a large study of 9989 patients showed that a multi-panel assay of stool DNA markers could detect colorectal cancer with a sensitivity of 92.3%, while 42.4% of patients with polyps were detected. The false positive rate of the DNA based test in this study was 13.4% (Imperiale et al., 2014), which would generate 134 false positives, and consequently 134 unnecessary follow-ups in the form of colonoscopy, for each 1000 patients screened.

Massive parallel sequencing has been proven successful in identifying mutations from cancerous tissues (Gerecke et al., 2013; Kinde et al., 2011) and specific cancer panels, such as the Ion AmpliSeq™Cancer Hotspot Panel v2 and the TruSeq Amplicon — Cancer Panel (TSACP) are now available for second generation platforms. However, despite covering a broad spectrum of genes, their sensitivity is bound at 5% (Frampton et al., 2013; Fang et al., 2013; http://www.edgebio.com/ion-ampliseq-fixed-panels-hct-15-colon-carcinoma-cell-line; Singh et al., 2013), a constraint which makes them ineffective when screening for CRC using stool DNA.

A recent survey of 224 CRC tumors by whole exome DNA sequencing (The Cancer Genome Atlas Network, 2012) shows that 93% of all tumors have mutations in the wnt signaling pathway, 62% have mutations in the RTK-RAS pathway and 61% have mutations in *TP53* signaling. The fraction of CRC tumors that will not have a mutation in any of these pathways is then $(1–0.93) \times (1–0.62) \times (1–0.61) = 0.6\%$, thus the theoretical false negative rate due to not looking for the driver mutations present in a tumor would be 0.6% if all possible mutations were screened in all of the genes involved in these pathways. The 15 amplicon assays described in this paper will not detect all mutations in these 3 key pathways, but is designed to detect the maximum number with a self-imposed limit of 15 amplicons. The number of amplicons in such an assay, i.e., the genomic spectrum that could be interrogated by deep sequencing, could certainly be enlarged, however, at the time of writing, a region of about 5000 nucleotide seemed to be the most appropriate projected trade-off between the size of the region to inspect and a cost-effective clinical setting, whereby sufficient sequencing data for a sample could be obtained by employing only on one SMRT cell.

The rationale behind such an assay is closely reinforced by the fact that, apart from the aforementioned genes, no equally informative biomarkers for CRC have been so far reported, as the cases of microRNA and gut microbiotas witness (Hrašovec and Glavač, 2012; Mazeh et al., 2013; Zhu et al., 2013; Dejea et al., 2013), where no definite conclusion can be drawn regarding an association between biomarkers of that type and the development of CRC.

In the current study, we present the first application of single molecule, real time, circular consensus sequencing (SMRT-CCS) (Travers et al., 2010) to the detection of mutations associated with CRC using stool DNA as analyte. The high quality of the raw sequence data produced by SMRT-CCS allows for a sensitivity of detection in the range of 0.5–2%, which is required to detect polyps using stool DNA as analyte, as most of the human DNA in a stool sample from such patients will be wild type (Traverso et al., 2002b).

2. Materials and methods

2.1. Experimental design

The main goal of this study is to test the specificity and sensitivity of third generation, single molecule sequencing in detecting mutated DNA at concentrations comparable to those observed in stool from exfoliated cells derived from early stage adenomas.

We performed a series of experiments to detect low frequency CRC mutations using an assay consisting of fifteen amplicons covering key regions of the genes most frequently mutated in CRC. The test sequence includes 8 overlapping amplicons covering codons 840–1581 of the APC gene, which is twice the size of the Mutation Cluster Region (MCR) covering codons 1210–1581 that has been used previously to detect mutations in stool DNA from patients with polyps (Traverso et al., 2002b). About 83% of APC mutations in sporadic CRC are found in the MCR,

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