MicroRNAs as molecular markers for screening of colon cancer.

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Abstract

The importance of melt curve analysis (MCA) is demonstrated by a foodomic study that we have carried out to facilitate interpretation of mild expression data, by measuring the magnitude of the expression of key micro(mi)RNA molecules in stool of healthy human adults that have been used as molecular markers, following the intake of Pomegranate juice (PGJ), functional fermented sobya (FS), rich in probiotic lactobacilli, or their combination. Total small RNA was isolated from stool of 25 volunteers, 20 to 34 years old, before and following a three-week dietary intervention trial. Expression of 88 miRNA genes was evaluated using Qiagen's 96 well plate RT2 miRNA qPCR arrays.

Keywords: Infective endocarditis, Mitral annular calcification, Methicillin-resistant *Staphylococcus aureus.*

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Description

Colon cancer (CC) could be cured at the early disease stage, and therefore screening is important for diagnosis, and potentially reduces mortality. Early detection would be desirable, if accurate and cost effective diagnostic methods are currently available, and would result in decreased disease mortality.

Current screening method, the immunological fecal occult blood test, FOBTi, lacks sensitivity and requires dietary restriction, which decreases compliance. Colonoscopy, considered as the "Gold Standard" for CC screening is invasive, and in certain cases could lead to mortality. Compared to the FOBT test, a non-invasive sensitive screen that does not require dietary restriction would be more convenient. Colonoscopy screening has been recommended for colorectal cancer (CRC). Although it is a reliable method to screen for that cancer, it is an invasive test, is often accompanied by abdominal pain, has potential for complications and is not economical, all factors that hampered its application worldwide.

On the other hand, a screening approach that uses the relatively stable and non-degradable microRNA molecules when extracted from either the non-invasive human stool, or the semi-invasive blood samples by available commercial kits and manipulated thereafter, would be more preferable than a transcriptomic messenger (m) RNA-, a mutation DNA-, an epigenetic- or a proteomic-based test. That approach utilizes reverse transcriptase (RT), followed by a modified quantitative real-time polymerase chain reaction (qPCR). To compensate for exosomal miRNAs that would not be measured, a parallel test could be performed on stool or plasma's total RNAs, and corrections for exosomal loss are made to obtain accurate results. Ultimately, a chip would be developed to facilitate diagnosis, as has been carried out for the quantification of genetically modified organisms (GMOs) in foods. If laboratory performance criteria are met, a miRNA test in human stool or blood samples based on high throughput automated technologies and quantitative expression measurements currently employed in the diagnostic clinical laboratory, would eventually be advanced to the clinical setting, making a noticeable impact on the prevention of colon cancer.

Stool testing has several advantages over other colon cancer screening media as it is truly noninvasive and requires no unpleasant cathartic preparation, formal health care visits, or time away from work or routine activities. Unlike sigmoidoscopy, it reflects the full length of the colorectum and samples can be taken in a way that represents both the right, as well as the left side of the colon. It is also believed that colonocytes are released continuously and abundantly into the fecal stream, contrary to situation in blood--where it is released intermittently-- as in FOBT, and transformed colonoctes produce more RNA than normal ones; therefore, this natural enrichment phenomenon partially obviate for the need to use a laboratory technique to enrich for tumorigenic colonocytes. Furthermore, because testing can be performed on mail-in-specimens, geographic access to stool screening is unimpeded.

It should be emphasized that although not all of the shed cells in stool are derived from a tumor, data indicate that diagnostic miRNA gene expression profiles are associated with adequate number of exfoliated cancerous cells and enough transformed RNA is released in the stool, and also the availability of measurable amount of circulating. miRNA genes in blood (either cellular or extracellularly), which can be determined quantitatively by a sensitive technique such as PCR in spite of the presence of bacterial DNA, non-transformed RNA and other interfering substances. That quantification is feasible because of the high specificity of PCR primers that are employed in this method, which overcomes all of these, stated obstacles; hence, the number of abnormally-shed colonocytes in stool, or total RNA presents in plasma or serum becomes unlimiting.

A test that employs miRNA in stool or blood could also result in a robust screen because of the durability of the miRNA molecules. Moreover, an approach utilizing *miRNA* genes is

more comprehensive and encompassing than a test that is based on the fragile messenger (m) RNA, for example, because it is based on mechanisms at a higher level of control. We believe that ultimately the final noninvasive test in stool or blood will include testing of several *miRNA* genes that show increased and decreased expression, and eventually a chip that contains a combination of these stable molecules will be produced to simplify testing.

For mature miRNAs testing, there are currently available commercial preparations that save time and provide the advantage of manufacturer's established validation and QC standards. Small noncoding RNAs that exhibit little variation in different cell types (e.g., snoRNAs and snRNAs) are poly-adenylated and are reverse transcribed (RT) in the same way as the small miRNAs and thereby could serve as controls for variability in sample loading and real-time RT-PCR efficiency. They are, however, not suited for data normalization in miRNA profiling experiments because they are not well expressed in serum and plasma samples. Therefore, normalization by a plate mean (i.e., mean C_T value of all the miRNA targets on the plate), or using a commonly expressed miRNA targets (i.e., only the targets that are expressed in all samples are used to calculate the mean value) would be needed for a proper normalization of the amplification reaction.

As colon cancer-specific miRNAs are identified in stool colonocytes or blood plasma by microarrays- and qPCR-based approaches as presented in this review, the validation of novel miRNA/mRNA target pairs within the pathways of interest could lead to discovery of cellular functions collectively targeted by differentially expressed miRNAs.

Unlike screening for large numbers of mRNA genes, a modest number of miRNAs is used to differentiate cancer from normal, and unlike mRNA, miRNAs in stool and blood remain largely intact and stable for detection. Therefore, miRNAs are better molecules to use for developing a reliable non-invasive diagnostic screen for colon cancer, since we found out that: a) the presence of *Escherichia coli* does not hinder detection of miRNA by a sensitive technique such as qPCR, as the primers employed are selected to amplify human and not bacterial miRNA genes, and b) the miRNA expression patterns are the same in primary tumor, or in diseased tissue, as in stool and blood samples. Although exosomal RNA will be missed when using restricted extraction of total RNA from blood or stool, a parallel test could also be carried out on the small total RNA obtained from non-invasive stool or semi-invasive blood samples, and the appropriate corrections for exosomal loss can then be made after the tests are completed.

To be able to screen several miRNA genes using PCR technology, a sequence-specific stem-loop RT primers designed to anneal to the 3'-end of a mature miRNA, followed by a SYBR Green®-based real-time qPCR analysis "TaqMan® PCR" method is often employed using an appropriate normalization standard. a "*reference*" housekeeping internal standard gene (e.g., endogenous reference genes *RNU6* genes *RNU6A* and *RNU6B, SNORD* genes *SNORD43, SNORD44, SNORD48, SNORA74A)* or miRNA normalizers (e.g., miRNA 16, miRNA-191), or in some cases against several standards because the total input amount may vary from sample to sample when doing relative quantification. To ensure that miRNA quantification is not affected by the technical variability that may be introduced at different analysis steps, synthetic nonhuman spike-in miRNA have been used to monitor RNA purification and RT efficiencies. In PCR reactions, strict QC procedures, as minimum information for publication of quantitative real-time PCR expression (MIQUE) are implemented in order to ensure the uniformity, reproducibility and reliability of the PCR reaction and data integrity.

Proper statistical analysis is needed for interpretation of data. Bioinformatics analysis can be employed to correlated miRNAs with mRNAs data.

Although methods that employ PCR in stool and blood samples are currently in the forefront of the quantitative methods to develop reliable screening markers, a chip that contain a combination of these genes could be produced to simplify testing, as has been accomplished in testing of genetically modified organisms in foods.

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