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Short Communication
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Rationale for Using of microRNA as Markers for Screening of Colon Cancer

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Colon cancer (CC) could be cured at early disease stage; therefore diagnostic screening potentially reduces mortality. Early CC detection is desirable, and would decrease mortality. Immunological fecal occult blood test (FOBTi) screening method lacks sensitivity and requires dietary restriction, which decreases compliance. Colonoscopy, considered as the "Gold Standard" for colorectal cancer (CRC) screening is invasive, and in certain cases could lead to mortality. Compared to the FOBT test, a noninvasive sensitive screen that does not require dietary restriction would be a more convenient test. Although colonoscopy screening is a reliable method to screen for colon cancer, it is an invasive test, is often accompanied by abdominal pain, and has potential for complications and is not economical; all are factors that have hampered its worldwide application.

On the other hand, a screening approach that uses the relatively stable and nondegradable micro(mi)RNA molecules when extracted from either the noninvasive human stool, or from 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-, or an epigenetic- or a proteomic-based test. The proposed quantitative miRNA 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 on stool or plasma's total RNAs needs to be carried out, and corrections for exosomal loss are made to ascertain accurate results. Ultimately, a chip need to 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 tests because: it is 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 and left side colon. Because 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 in stool 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 therefore unimpeded, which makes it prepherable to screening in blood.

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. Moreover, the availability of measurable amount of circulating miRNA genes in stool or blood (either cellular or extracellular), by a sensitive quantitative technique such as PCR in spite of the presence of bacterial DNA, non-transformed RNA and other interfering substances, makes accurate quantification of miRNAs feasible, because of the high specificity of PCR primers that are employed in this method, will overcomes screening obstacles; hence, the number of abnormally-shed colonocytes in stool, or total RNA present in stool, 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,

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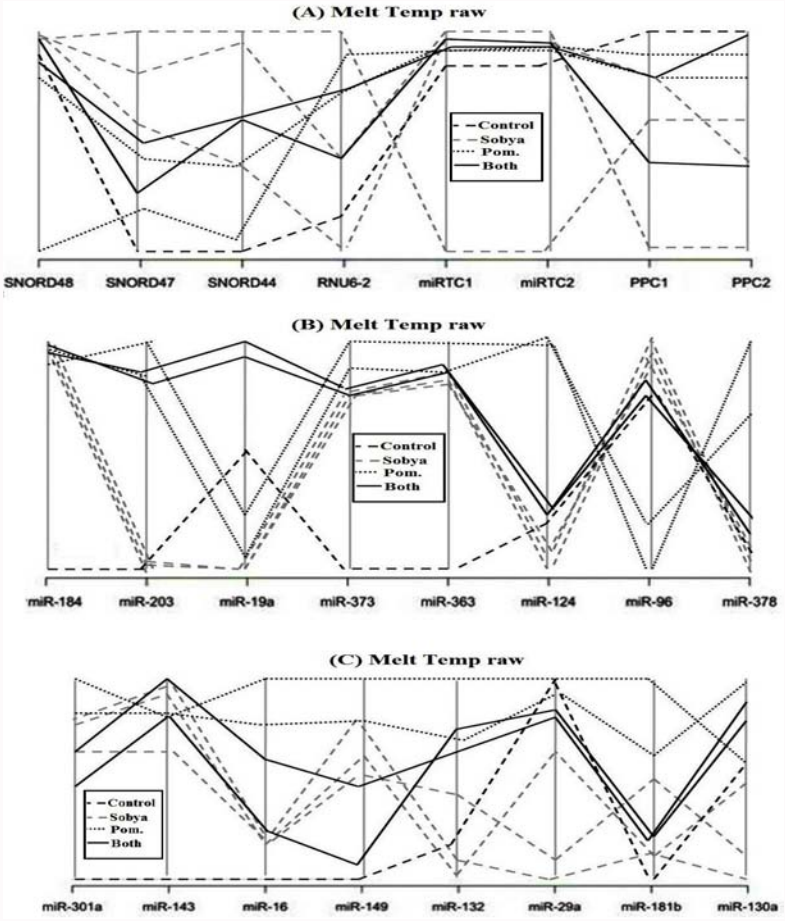

Figure 1: The genes were ordered using the *p*-values of a one way ANOVA based on groups. Genes with the smallest *p*-values are presented first.

because it is based on mechanisms at a higher level of control. I 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 containing 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 polyadenylated 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 in blood because they are not well expressed in serum and plasma samples. Therefore, normalization in blood by a plate mean (i.e., mean C_T value of all the miRNA targets on the plate), or by 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 by microarrays- and qPCR-based approaches, the validation of novel miRNA/mRNA target pairs within 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 noninvasive diagnostic screen for colon cancer, since: 1) 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 2) 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 run on the small total RNA obtained from noninvasive stool or seminvasive blood samples, and the appropriate corrections for exosomal loss can then be made after the tests are carried out.

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. 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 can be used to monitor RNA purification and RT efficiencies. In PCR reactions, QC procedure, as Minimum Information for Publication of Quantitative Real-Time PCR expression (MIQUE) is implemented to ensure uniformity, reproducibility and reliability of the PCR reaction and data integrity. Statistical analysis is used for data interpretation, and bioinformatics may be employed to correlated miRNAs with mRNAs data. Our group demonstrated using melt curve analysis (MCA) for interpretation of mild nutrogenomic miRNA expression data in a recent three week dietary intervention trial, by measuring the magnitude of the expression of key miRNA molecules in stool of healthy human adults as molecular markers, following the intake of Pomegranate juice (PGJ), functional fermented sobya (FS), rich in potential probiotic lactobacilli, or their combination, in which total small RNA was isolated from stool of 25 volunteers before and following dietary intake. Expression of 88 miRNA genes was evaluated, using Qiagen's 96 well plate RT^2 miRNA qPCR arrays. Employing parallel coordinate's plots, no observed significant separation was observed for the gene expression (Cq) values, using Roche 480® PCR Light Cycler instrument, and none of the miRNAs showed significant statistical expression after controlling for the false discovery rate. On the other hand, melting temperature profiles produced during PCR amplification run, identified seven significant genes (miR-184, miR-203, miR-373, miR-124, miR-96, miR-373 and miR-301a), which separated candidate miRNAs. These miRNAs could thus function as novel molecular markers of relevance to oxidative stress and immunoglobulin function, for the intake of polyphenol (PP)-rich, functional fermented foods rich in lactobacilli (FS), or their combination see (Figure 1), which is a graphical representation of the parallel plot coordinates of the studies miRNA genes. The genes were ordered using the *p*-values of a one way ANOVA based on groups. Genes with the smallest *p*-values are presented first. (Figure 1A) below shows control genes, while in (Figures 1B and C) five miRNA genes show separation.