An improved method to detect low levels of methylated DNA

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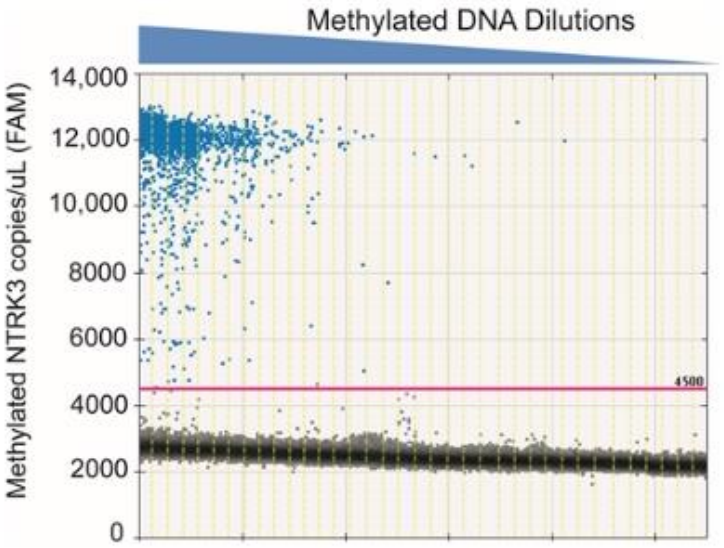

Detection of decreasing amount of methylated NTRK3 in serial dilutions of methylated and unmethylated DNA standards containing 100%, 20%, 4%, 0.8%, 0.16%, 0.032%, 0.006% and 0% of methylated DNA (20 ng DNA/sample), as shown by ddPCR analysis. Each sample was partitioned into an average of 15,000 droplets per well and replicated in 4 wells. The droplet counts (positive and negative) from all 4 replicates were combined for the final analysis. An event with fluorescence amplitude value >4,500 was considered a methylation-positive event (red line). The number of methylation-positive events decreased as the methylated DNA was progressively diluted with unmethylated DNA. Using ddPCR, it was feasible to detect and absolutely quantify as low as 0.032% of methylated NTRK3

Image provided by Dr. Ming Yu

The addition of methyl groups to DNA is a process that plays an important role in development and in certain diseases, through altering gene expression in cells. Abnormal DNA methylation patterns - either higher or lower levels compared with normal tissue - are associated with many human malignancies, including colorectal cancer. Interestingly, these alterations have been detected not only in colon cancer and colon polyps, the precursors to colorectal cancer, but also in the normal colon tissue in individuals with concurrent colorectal cancer, who are at high risk of colon polyps and metachronous (not synchronous) colorectal cancer. Thus, there is potential for methylated genes to be used as biomarkers for early detection of colorectal cancer. There is a problem, however: the aberrations are present at very low levels, and cannot be easily detected using current polymerase chain reaction (PCR) technologies. Dr. Ming Yu in the laboratory of Dr. William Grady in Fred Hutch's Clinical Research Division accepted this challenge, and set out to develop a more precise and sensitive method for detecting methylated DNA. In the September issue of *Epigenetics*, Drs. Yu, Grady, and colleagues describe their efforts to develop this method and their resulting conclusions.

The investigators based their innovation on a conventional methylation-specific PCR method called MethyLight, but added the benefits of droplet digital PCR (ddPCR) - a new technology that enables precise detection of nucleic acid targets in clinical samples. Using colon cancer tissue samples and colon mucosa biopsy samples, the technique was tested for detecting infrequently methylated alleles - an application of ddPCR that had not been previously published. "In our study, we developed a ddPCR version of MethyLight assays and demonstrated that the resulting MethyLight ddPCR offered improvements in sensitivity with a much lower limit of detection, improved absolute quantification of methylated alleles, as well as increased precision in detecting infrequently methylated targets," Dr. Yu said.

The investigators used the BioRad QX200 ddPCR system, which partitions each 20-µL PCR reaction into an average of 15,000 nanoliter droplets through water-oil emulsion. Essentially, this enables 15,000 simultaneous independent PCR reactions, with each droplet PCR-cycled to endpoint and read with a two-color fluorescence reader. Thus, it is possible to determine how many droplets are positive. Compared with conventional MethyLight PCR, the respective limits of quantification and detection were 25 and 20 times lower with MethyLight ddPCR, as determined through comparative analysis of methylated *EVL* and *NTRK3* genes. Where the conventional technique was able to detect 1 methylated *NTRK3* allele in a background of 125 unmethylated alleles, the new method detected 1 out of 3125. Moreover, low levels of *EVL* methylation were not detected in either of nine samples of normal colon mucosa from people with colorectal cancer using conventional MethyLight PCR, but MethyLight ddPCR selectively detected and absolutely quantified methylated *EVL* in all nine samples.

The detection of infrequent methylation events in various clinical specimens is crucial, both for identifying cancer-associated epigenetic alterations that may be risk biomarkers, and for the early detection of colorectal cancer using blood or stool samples. "The ultimate goal for implementation of a sensitive detection tool like ddPCR Methylight is to determine its ability to detect methylated DNA in body fluid samples like blood, urine, or stool and to study normal tissue for evidence of field cancerization," explained Dr. Yu, who believes that ddPCR MethyLight is well suited to address many of the technical challenges associated with these applications, such as contaminating background DNA or the presence of various DNA polymerase inhibitors.

With this study, the investigators have demonstrated proof of principle of the technique, which is currently being validated through further assessment of other risk biomarker candidates. Dr Yu is optimistic: "It will improve the sensitivity and precision for detecting infrequently methylated genes and will allow us to conduct studies to determine if methylated genes can be used as colon cancer

risk biomarkers." In the long run, these biomarkers could facilitate and improve the effectiveness of personalized cancer prevention programs, hopefully decreasing the mortality of colorectal cancer - currently the second leading cause of cancer-related death in the United States.

Yu M, Carter KT, Makar KW, Vickers K, Ulrich CM, Schoen RE, Brenner D, Markowitz SD, Grady WM. 2015. MethyLight droplet digital PCR for detection and absolute quantification of infrequently methylated alleles. *Epigenetics*. 10(9):803-9.

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