FEATURE

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Direct Quantitative Analysis of Multiple miRNAs (DQAMmiR) – a New Tool for Personalized Cancer Medicine

CANCER IS A HETEROGENEOUS DISEASE - not only do different types of cancer differ from each other, but individual cancers of the same type also vary from patient to patient.

Therefore, the classical "one-size-fits-all" approach in cancer treatment is not efficient. The base paradigm of personalized cancer medicine is that patient-specific characteristics of cancer must be used in choosing a therapy to make it efficient. Molecular markers in tumours are the most reliable means of characterizing individual cancers and microRNA (miRNA) is, in turn, among the most promising types of molecular markers.

MiRNAs are 18 to 25-nucleotide-long strands of RNA that play an important role in regulation of cellular processes. Abnormal production of miRNA in cells was implicated in cancer suggesting that miRNA can be used for cancer diagnosis and therapy guidance. tumourigenesis typically leads to altered levels of several miRNAs, for example, abnormal production of two miRNAs was linked to colorectal cancer in humans. Accurate molecular characterization of individual cancers requires identification of miRNA signatures of cancer, which are per se quantitative levels of multiple miRNAs. Hundreds of candidate miRNA markers of cancer have been already identified by comparing cancerous and normal tissues with microarrays which allow profiling thousands of miRNAs in a semi-quantitative fashion. Despite these efforts, currently there is no single miRNA signature validated for cancer prognosis and therapy guidance. Validation of miRNA signatures is the main obstacle to using miRNA in personalized cancer medicine, and the lack of a suitable validation technology for miRNA signatures is arguably the major challenge.

To validate a miRNA signature, one needs to accurately measure levels of multiple miRNAs in order to then establish the correlation of these levels with clinical outcomes of cancer for a statistically significant population of patients (as many as tens of thousands of patients). Accordingly, a suitable validation technology must be: (i) quantitative, (ii) highly-sensitive, (iii) rugged, and (iv) capable of simultaneously analyzing multiple miRNAs. Quantitation is required since cancer sub-types may differ from each other in varying quantities of miRNAs in the molecular signature. High sensitivity is required to allow analyses of very small tissues samples; for instance, those from fine-needle aspiration biopsies or single cells. Ruggedness is needed to ensure that data obtained in different labs with different instruments are comparable (the effort of many labs is needed to process a very large number of samples required for validation). Finally, the simultaneous analysis of multiple miRNAs is needed to generate uniform signatures as well as reduce cost of analysis, thus, making validation of miRNA signatures feasible.

Most available methods of miRNA detection are indirect (e.g. quantitative reverse-transcriptase polymerase chain reaction, microarrays, surface plasmon resonance, next generation sequencing, etc.); they require chemical or enzymatic modifications of miRNA prior to the

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analysis. Modifications make the analysis cumbersome and lead to reduced accuracy due to different efficiencies of modifications for different miRNAs. All indirect methods do not satisfy more than one of the four conditions specified above for a suitable validation technology. A few direct methods, which do not require any modification of the target miRNA, are available: Northern blotting, signal amplifying ribozymes, in situ hybridization, bioluminescence detection, and two-probe single-molecule fluorescence. However, they have limitations – the first three are only semi-quantitative when used for multiple miRNAs while the latter two can hardly be used for multiple miRNAs.

Direct Quantitative Analysis of Multiple miRNAs (DQAMmiR) that has a potential to satisfy all four requirements has been recently developed by a group of researchers from York University upon a classical hybridization approach.[1] In this approach, hybridization probes fluorescently labeled for detection and taken in excess to miRNAs, bind miRNAs sequence-specifically, and unreacted probes are separated from the probe-target hybrids by capillary electrophoresis and detected with laser-induced fluorescence. The challenging problems of electrophoretic separation of (i) the unreacted probes (single strand DNA) from the probe-miRNA hybrids and (ii) the hybrids from each other were solved through the combined use of single strand DNA binding (SSB) protein in the electrophoresis run buffer, and drag tags on the probes which change the probe electrophoresis mobility.

Figure 1 schematically illustrates the concept of DQAMmiR. In the hybridization step, the excess of the probes of concentrations [P]0,i is mixed with the miRNAs to be analyzed, which leads to all miRNAs' being hybridized but with some probes left unreacted. A short plug of the hybridization mixture is introduced into a capillary filled with an SSB-containing run buffer. Due to its unique binding selectivity, SSB binds all unreacted ssDNA probes but does not bind the double stranded miRNA-DNA hybrid. When an electric field is applied to the ends of the capillary, the SSB-bound probes move faster than all the hybrids (SSB serves the role of the propellant). The miRNA-DNA hybrids have similar lengths and cannot be easily separated by electrophoresis; however, different drag tags on the probes make different hybrids move with different velocities. SSB-bound probes will move with similar velocities if the drag tags are small with respect to SSB. In such a case, a fluorescent detector at the end of the capillary generates separate signals for the hybrids and a cumulative signal (one peak or multiple peaks) for the unreacted probes. The amounts (or concentrations) of miRNAs in the sample are finally calculated with a simple mathematical formula that uses the integrated signals which correspond to peak areas in the graph: AH,i for the hybrids and AP for a sum of all unreacted probes.

DQAMmiR is quantitative and accurate. It does not involve amplification or chemical/enzymatic modifications

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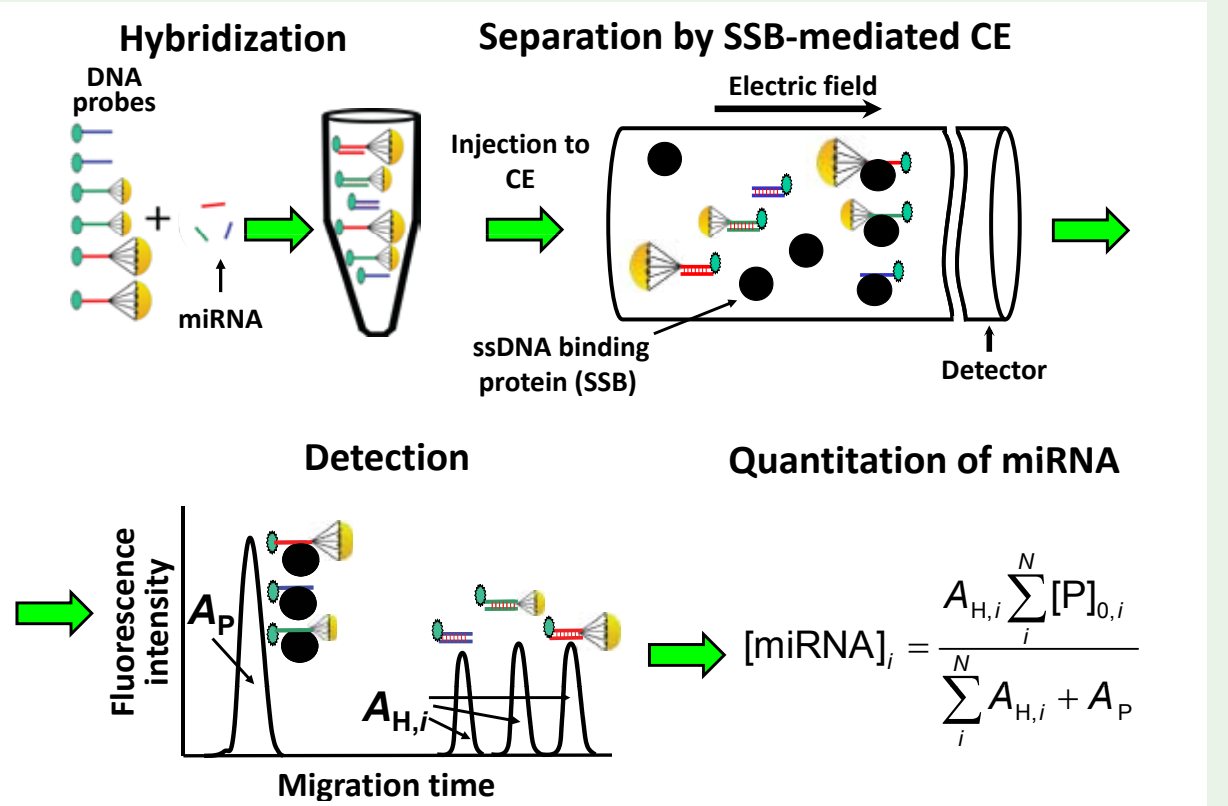
While the ruggedness of DQAMmiR has not yet been confirmed experimentally, one can expect the method to be rugged. DQAMmiR is a calibration-free method that can determine absolute amounts of miRNA. The results do not depend on an instrument used provided that it has a suitable sensitivity.

Figure 1



Schematic illustration of the direct quantitative analysis of multiple miRNAs (DQAMmiR) by CE with fluorescence detection. miRNAs and their complimentary ssDNA probes are shown as short lines of the same color, drag tags are shown as parachutes, a fluorescent label is shown as small green circles and ssDNA-binding protein (SSB) is shown as large black circles.

that could affect accuracy of quantitation. The dynamic range of DQAMmiR is easily adjusted by changing the concentrations of the hybridization probes. Not only is the DQAMmiR quantitative but it also allows one to determine absolute amounts of miRNAs in the sample by using the unreacted probes as a reference standard. This internal reference standard makes DQAMmiR calibration-free which is the method's unique and powerful feature. DQAMmiR can, thus, be used for distinguishing miRNA signatures that differ in miRNA amounts.

With regards to DQAMmiR sensitivity, commercial capillary electrophoresis equipment can only facilitate detection limits of approximately 300,000 copies of miRNA in a consumed sample. With this relatively high detection limit DQAMmiR would be inapplicable to measuring miRNAs in fine-needle aspiration biopsies, which became standard in diagnosis of cancer and other diseases. Indeed, as few as 1,000 copies of miRNA may be present in a single cell, and fine-needle biopsies typically contain in the order of 100 cells. The total number of miRNA copies in such a small sample would be less than 100,000, which is below the DQAMmiR detection limit possible with commercially available CE instrumentation. To solve this problem, the researchers from York University collaborated with Prof. Gradinaru's group at the University of Toronto, Mississauga, to develop ultra-sensitive capillary electrophoresis (CE) with confocal time-resolved fluorescence (CTRF) detection through an embedded capillary interface (ECI).[2] CE-CTRF-ECI decreased the limit of detection to 1,000 copies of miRNA, and further improvement can be achieved with more advanced prototypes of CE-CTRF-ECI instrumentation. Importantly, instrumentation for CE-CTRF-ECI is not only sensitive but also robust; it does not include sophisticated custom-made components that are easy to align and keep alignment. DQAMmiR performed with CE-CTRF-ECI can, thus, be used for analysis of miRNAs in a small number of cells and even in single cells.

While the ruggedness of DQAMmiR has not yet been confirmed experimentally, one can expect the method to be rugged. DQAMmiR is a calibration-free method that can determine absolute amounts of miRNA. The results do not depend on an instrument used provided that it has a suitable sensitivity. Moreover, DQAMmiR's performance is not significantly dependent on the sample matrix and even a crude cell lysate can be sampled for the analysis. This suggests that the method has minimal susceptibility to variations in sample preparation procedures, thus allowing different laboratories and operators to obtain comparable data. This in turn will facilitate screening large populations of patients required for detailed profiling of cancer sub-types.

DQAMmiR is capable of simultaneously analysing multiple miRNAs. The number of miRNAs that can be run together is limited by the number of hybrid peaks that can be baseline-separated in the time window between a peak of miRNA hybrid with untagged probe and a peak of SSB-bound unreacted probe (see Fig. 1). The current estimate suggests that simultaneous analysis of 10 different miRNAs should be easily achievable. This number can be further increased by a factor of 2 or three if two or three different fluorophores are used as labels along with two or three spectral channels in the instrument.

While being potentially suitable for validation of miRNA signatures of cancer, DQAMmiR still requires significant research effort to become a practical tool in personalized cancer medicine. A generic approach to the design of multiple hybridization probes with different drag tags is still to be developed. For DQAMmiR to be used by multiple groups, commercial instrumentation for CE-CTRF-ECI must be available. Finally, DQAMmiR still need to be rigorously validated before it can be widely used in laboratory and clinical settings. The proof of principle for DQAMmiR and encouraging initial results certainly stimulate the effort towards making it a practical research and clinical tool.

References:

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2. Dodgson, B.J.; Mazouchi, A.; Wegman, D.W.; Gradinaru, C.C.; Krylov, S.N. Detection of a thousand copies of miRNA without enrichment or modification. Submitted to Analytical Chemistry on June 5, 2012.

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