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Aptamer-Facilitates Biomarker Discovery

By Sergey N.Krylov

Biomarkers are molecular signatures associated with the quantity of molecules in the cells or their state (conformation, association with other molecules, or localization). Biomarkers often serve as surrogate endpoints in pharmaceutical drug trials and can also be used as quantifiable indicators of cellular states in screening for diseases and guiding medical treatments. The importance of biomarkers is widely recognized and academic and industrial R&D in this area has been persistently growing in the past decade. Despite the significant efforts and investments, relatively few biomarkers are used in clinical practice; moreover, the rate of their introduction in practice is reducing. One of the major reasons for this is the lack of efficient methods for biomarker discovery.

Conventional methods for biomarker discovery include mRNA screening (with quantitative PCR or hybridization arrays) and 2-dimensional gel electrophoresis combined with mass spectrometry. While being the work horse in biomarker discovery, these methods share several important limitations. First, they require destroying the cells prior to the discovery process, thus, making it impossible to discover biomarkers associated with the state of molecules (conformation, association with other molecules, or localization). Second, the conventional methods are prone to false positive and false negative results. Third, even when biomarkers are discovered, their practical use still requires affinity probes (usually antibodies) the development of which can be a lengthy process.

York Chemical Cytometry Laboratory (YCCL) has recently introduced a biomarker discovery technology, which addresses the three above problems and promises to speed up the discovery of biomarkers and their progress to practical use. The technology is called aptamer-facilitated biomarker discovery (AptaBiD) and combines two processes: biomarker discovery and development of affinity probes for them. The two processes cannot be separated, however, as biomarkers are discovered through the development of affinity probes to them. As affinity probes, the method uses DNA aptamers (aptamers for short).

Aptamers are single stranded DNA molecules capable of binding their molecular targets with high selectivity and affinity. Due to this unique ability, aptamers are often viewed as artificial antibodies and proved potent as therapeutics and affinity probes in molecular diagnostics. In contrast to antibodies, aptamers are developed in an artificial selection process which can produce aptamers for molecular targets on intact cell in their native states.

AptaBiD requires two cells populations: a population of “target cells” and a population of “non-target cells”. Biomarkers are molecular signatures of target cells which are not present on non-target cells. The AptaBiD technology involves three major stages: (i) differential multi-round selection of aptamers for

biomarker of target cells; (ii) aptamer-based isolation of biomarkers from target cells; and (iii) mass spectrometry identification of biomarkers. First, aptamers are selected from a combinatorial library of DNA including as many as 1015 different DNA sequences (naive library); every sequence in such a library is statistically unique. The library is mixed with non-target cells and incubated to allow for DNA to bind molecules on cell surface. The cells are spun down
along with the binders and discarded; in this step non-specific cell binders are removed. The non-binders are collected, amplified by polymerase chain reaction (PCR), and mixed with target cells and allowed to bind them. Target cells are spun down along with DNA bound to them. The supernatant is discarded, while DNA is dissociated from the cells and amplified by PCR. DNA which binds target cells but does not bind non-target cells is assumed to bind biomarkers of target cells. The described differential selection procedure is repeated 10 times to gradually generate a pure pool of aptamers capable of binding biomarker of target cells. Second, the aptamer pool is used to isolate biomarkers from target cells. Biotin is covalently linked to aptamers. Biotinilated aptamers are reacted with target cells and allowed to bind biomarkers. Magnetic beads with streptavidin immobilized on their surface are added to the cell-aptamer mixture. Biotin tightly binds
to streptavidin and links biomarkers on cells to magnetic beads through aptamers and biotin-streptavidin bridge. The cells are lysed by a soft lysing agent not to destroy the
conjugates. The conjugates are kept on the surface of the vial with a magnet under the vial while the cell debris is washed away. The biomarkers are dissociated from the aptamer with urea and collected with the supernatant when the aptamer-biotinstreptavidin-bead complexes are kept by the magnet on the vial surface. The collected sample contains purified biomarkers that differ target cells from non-target cells. Third, the collected biomarkers are identified. Apta-Bid in its current format aims
at the discovery of protein biomarkers. The protein in the sample of biomarkers is trypsinized to make their short fragments. The fragments are then separated by liquid chromatography and analyzed by mass spectrometry. The identified fragments are searched in a protein database such as MASCOT to identify the protein biomarkers.

While aptamers constitute a highly efficient approach to the isolation of protein biomarkers, false positive molecular targets are possible. To eliminate false positive targets, AptaBid uses a series of “filters”. First, only proteins with “high” and “very high”
confidence of mass spectrometry identification are considered. Then, the biotin-labeled
naive library (instead of an aptamer pool) attached to streptavidin-coated magnetic beads is used in negative control experiments to eliminate DNA-binding and bead-binding proteins. Further, is cellsurface biomarkers are to be discovered, non-membrane proteins are eliminated from the protein list. The experiments are repeated at least three times and only proteins that appeared in all experiments are left in the list of biomarkers. In addition, proteins that are cross-listed for target and non-target cells are eliminated
to give the finest list of biomarkers that so far can be discovered with AptaBiD.

AptaBiD uses aptamer pools to discover biomarkers but does not require the identifi-cation of aptamers in the discovery process. Using entire aptamer pools has several advantages. First, sequencing aptamers is not a part of AptaBiD which makes the procedure simples and less expensive. Second, all “aptamerogenic” biomarkers can be discovered using entire pools while only a fraction of biomarkers can be discovered when individual aptamers are used. Finally, using pools can facilitate linking a cell to a magnetic bead through a number of biomarkers thus making think binding strong to survive the washing procedures. When individual aptamers are used a cell will likely be bound to a magnetic bead through a single biomarker-aptamer complex; such a complex may not be strong enough to maintain the complex during the vigorous washing procedures.

While sequencing aptamers is not a part of AptaBiD, aptamers must be sequenced if they are to be used for detecting a specific biomarker. As affinity probes, aptamers have a number of advantages over antibodies. When aptamer sequence is known, aptamer can be synthetically produced in large amounts. Aptamers can be fluorescently labelled at the end of DNA strand without affecting its binding to the target. Aptamers are not immunogenic and if protected can be left for hours in biological fluids. Thus, aptamers can be injected in the organism to track biomarkers in vitro. As was mentioned above, conventional biomarker discovery technologies are destructive in their nature. This imposes a serious

problem for the use of biomarkers discovered with them. When biomarkers have been discovered with the destructive approaches, affinity probes are developed for such biomarkers using the purified biomarker taken out of the cellular context. Even if the probe is developed it can likely be unable to bind the biomarker in its native state on the cell. This can dramatically limit the usefulness of biomarkers discovered with traditional destructive techniques. AptaBiD, in contrast, discovers biomarkers in their native states. Aptamers are developed to proteins on cells and therefore can be immediately used to detect the presence of the protein on the intact cell using cytometry techniques, such as flow cytometry or image cytometry (microscopy).

Conventional destructive approaches are prone to false-positive and false-negative results. False positive ones originate from intensive sample processing (cell lysis, isolation and purification of RNA and proteins, trypsin proteolysis) preceding the biomarker identification step. Even very small differences in sample processing between the target cells and non-target cells can lead to apparent differences between some proteins, which can be mistakenly identified as biomarkers. False positive biomarkers can also originate from detecting false differences in low abundant proteins if their amount is at the instrument detection limit. On the other hand, the “linear” nature of biomarker “detection” in conventional tehniques can overlook proteins which are expressed in target cells and non-target cells at close amounts but localized and post-translationally modified differently. This later lead to false-negative results in biomarker discovery.

AptaBiD helps to reduce the yield of false-positive and false-negative results. It is based on multi-round selection of aptamers for differential molecular targets on the cells which facilitates “exponential detection” of biomarkers (in contrast to “linear detection” in the conventional approaches). The multiple rounds suppress stochastic variations in cell populations and unintended differences in cell processing, thus, reducing the chance of false positive results. The “exponential detection” of biomarkers allows for sensing minor differences in molecular targets between the two cell populations if the differences persist from round to round of aptamer selection. For example, the detection ability for a persistent difference of 2 times in the biomarker amount could be improved by a factor of 2N-1 when taken through N rounds of aptamer selection.

Major equipment, which is required for AptaBiD, includes a flow cell analyzer (flow cytometers), a centrifuge, a PCR thermocycler, and a mass spectrometer. When the populations of target and non-target cells are contaminated, fluorescence-activated cell sorting (FACS) can assist aptamer selection.

To conclude, AptaBiD provides a number of advantages over conventional approaches to biomarker discovery. While it is still in its infancy it promises to speed up the biomarker discovery process along with the clinical use of biomarkers for diagnostic and therapeutic applications.

References:

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