October 26, 2019

White Paper

Proteomics as Compared with Circulating Tumor DNA for Early Stage Cancer Detection (Screening)

Recent work with circulating tumor DNA and various new approaches to proteomics where certain protein markers active in the tumor microenvironment are used along with, so-called "Proteomic Noise Suppression", have shown utility in detection of cancer. These recent developments have produced data about efficacy of both methods for detecting early stage tumors with blood tests and especially how viable these methods are for early stage detection and cancer screening. This White Paper will briefly discuss the state of the art for both methods. In general, we find that the mechanism that limits detection of these methods are fundamentally different. The proteomics method seems to have a fundamental advantage because the starting point is immune response which corresponds with very early stage tumor formation.

Circulating Tumor DNA

Circulating tumor DNA methods detect several types of tumor related abnormalities found in or associated with tumor DNA. These abnormalities are tumor-specific and presumably are related to the abnormal and malignant behavior of the tumor. The assumption is that these abnormal DNA are sloughed off into the blood and are detectable with an assay.

These tumor related changes fall into two categories, 1) direct mutant DNA sequences, one or more bases are divergent from normal; 2) errors in the DNA epigenomes for the tumorous tissue. This second category has several types, DNA methylation being the most studied with regard to cancer related cell abnormalities. In both cases the assay for detection of these modified DNA's require; 1) DNA extraction from the test sample matrix whole blood. The extraction will contain both types of abnormal DNA and a vast excess of normal human DNA (from white blood cells). Next the Abnormal tumor related DNA must be amplified by the polymerase chain reaction (PCR) to amplify the abnormal DNA such that it can be detected over and above the level of normal DNA. There are differing PCR reactions for each type of DNA specific to the assay mutant or abnormal methylation. Both assays are limited in detection by the same phenomena, background amplification of normal DNA by the mutant or tumor methylated DNA primer.

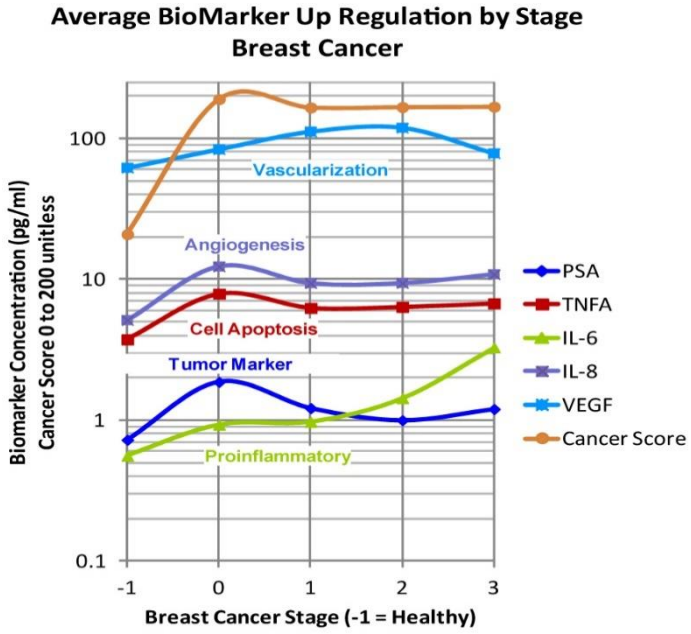
In either case, the PCR primer that starts the amplification process has a finite but statistically low chance of annealing to normal DNA --- thus starting a false positive amplification. The final result --a true positive result -- must be such that the detected tumor related DNA be above the level of the assay "blank" or "zero" result from samples run that do not possess any the of tumor related DNA. So called normal samples, without disease are run in replicates, the so called zero calibrator. A positive result, ctDNA positive, then must have signal above the "zero" to be statistically true. The referenced paper[1] surveys four different ctDNA assays and reports that a minimum tumor size of about 1 centimeter or more is required for the signal to exceed the blank. In other words, the ability of the ctDNA assay to detect a true positive is directly related to the tumor size and is limited for small early stage tumors.

[1] **Early Stage NSCLC – Challenges to Implementing ctDNA based Screening and MRD Detection ---Abbosh, Birbak and Swanton; Nature 2018**

Extensive testing for methylated DNA in a pan cancer test[2] shows that, indeed, detecting methylated DNA seems to be able to correctly identify the Tissue of Origin for multiple cancer types for stage II and higher cancers. However, the sensitivity, at 99% specificity, was poor for 12 specified stage I cancers ranging from 27% to 43%.

Another methylation based test for liver cancer marketed as an "LDT" test by the Laboratory for Advanced Medicine reports 80% sensitivity at 86% specificity. This test is currently in clinical trials and no independent third party results are available.

Proteomic Noise Suppression based on Tumor Microenvironment Active Proteins. The referenced White Paper[3] (May 15, 2017), describes a method where tumor microenvironment (TME) active proteins, cytokines, when used as cancer biomarkers coupled with a novel method for processing the raw serum concentration measurements can produce very high predictive power and superior detection of early stage 1 or even stage 0 cancer. Breast cancer pre-clinical work done at the Gertsen Institute, Moscow, shows 100% correct detection of 71 stage 0 and 1 samples of a total 198 samples breast cancer positive samples. Note that a stage 0 breast cancer tumor is less than 2 mm in size, far smaller and earlier in development than the 1 cm tumor required for ctDNA detection. The overall sensitivity of the blinded trial was 97% and the specificity was 96.6% for stages 0 through 4. Similar results were found in the non-small cell lung cancer pre-clinical trials showing 100% accuracy of NSCLC stage one samples and the overall trial showed 99% sensitivity and 98% specificity.



The graph to the left shows the actions of these TME active proteins. Notice that at the onset of stage 0 breast cancer (from healthy -1), the immune system biomarkers show a marked upward spike. As the tumor progresses through stage 1 to 3 these biomarkers are suppressed as the tumor begins to secrete anti-inflammatory cytokines. Also, the tumor markedly secretes angiogenesis cytokines to increase circulation in surrounding tissue at stage 0. This local tissue angiogenesis is supplanted by vascularization of the bulk tumor in later stages as the tumor grows. This same behavior is seen in ovarian, prostate, and ovarian cancers. Notice that these serum concentration measurements are well above the assay limit of detection and are in the regions of the assay where the signal is strongest. Thus the best measurements are available at the earliest stage of cancer.

Assay Limit of Detection versus Cancer Stage

In the case of ctDNA cancer detection, the limiting factor is the number of ctDNA fragments that slough off into the blood, as measured against the PCR assay limiting zero background signal. This parameter, the zero

[2] Genome-wide Cell-free DNA (cfDNA) Methylation Signatures and Effect on Tissue of Origin (TOO) Performance; Minetta C. Liu, MD; Arash Jamshidi, PhD; Oliver Venn, DPhil; Alexander P. Fields, PhD; M. Cyrus Maher, PhD, MS, MPH2; Gordon Cann, PhD; Hamed Amini, PhD; Sam Gross, PhD; Joerg Bredno, PhD; Meredith Halks- Miller, MD; Jan Schellenberger, PhD; Kathryn N. Kurtzman, MD; Eric T. Fung, MD, PhD; Tara Maddala, PhD; Geoffrey R. Oxnard, MD3; Eric A. Klein, MD; David R. Spigel, MD; Anne-Renee Hartman, MD; Alexander M. Aravanis, MD, PhD; Michael V. Seiden, MD, PhD; Mayo Clinic, Rochester, MN. GRAIL, Inc., Menlo Park, CA.; Dana Farber Cancer Institute, Boston, MA. Cleveland Clinic, Cleveland, OH. Tennessee Oncology, Nashville, TN. US Oncology Research, The Woodlands, TX. Corresponding author: liu.minetta@mayo.edu

[3] Diagnostics Proteomics – A New Approach – White Paper" May 15, 2017,is available from OTraces, Inc. www.otraces.com

background, will be very difficult to reduce as it is driven by the statistical thermodynamics of the annealing characteristics of the normal human DNA to the mutant PCR primer. It appears that the tumor must be at least 1 cm is size or larger to produce enough ctDNA to see above this background.

In the case of Proteomics coupled with our Noise Suppression and spatial proximity correlation methods, the detectability of early stage cancer is driven by the clustering characteristics of the noise suppressed concentration measurements in the multi-dimensional orthogonal correlation matrix where the proximity correlation is done (see reference 3). As the actions of the TME active proteins is the strongest at stage 0 and stage 1, this separation is the clearest at early stage, after which a few false negative and positive results show up as the immune system is now being suppressed by the tumor (but counter-acted by the tumor vascularization). In other words, tumor size is irrelevant and the early stage actions of the immune system drives detection. The immune system "sees" the small tumor at early stages far better than at later stages as the tumor tends to suppress the immune actions as the tumor progresses.

Overall, the mechanism that limits detection of these methods is fundamentally different and the proteomics noise suppression method seems to have a deep fundamental advantage.

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