National Cancer Institute

The Early Detection Research Network (EDRN)

Scientific Research Highlights

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Executive Summary

The development of new biomarkers for early cancer detection that can change clinical practice and ultimately have an impact on overall survival and mortality from the disease is a lengthy process that begins with the discovery of promising candidate biomarkers, rigorous validation, and implementation in the clinic. The success of this process requires a complex, dedicated infrastructure that facilitates the coordination, management and collaboration among many institutions, both from academia and industry, with the involvement of scientists and clinicians with diverse expertise. The Early Detection Research Network (EDRN) has fulfilled these expectations by establishing an infrastructure and a process for biomarker development using a multidisciplinary and multi-institutional approach. This infrastructure, combined with the development of a highly interactive biomarker knowledge system of integrated databases and informatics tools through EDRN's collaboration with the Jet Propulsion Laboratory (JPL), serves as a model for the conduct of translational biomarker research, which is clearly aligned with the goals and objectives of the National Cancer Institute (NCI) and the broader National Institutes of Health (NIH) community.

Since its inception, the main focus of the EDRN has been to bring new biomarkers to clinical validation. Early on, EDRN investigators recognized that the biomarker field was quite nascent, and consequently took on the responsibility to establishing guidelines for a phase-based biomarker development, as well as study design criteria for rigorous clinical validation. These have now been well accepted and adopted by the biomarker research community at large.

Over the past five years, EDRN investigators invested significant efforts for enriching the biomarker development pipeline to address significant unmet clinical needs in the early detection of cancer. These efforts have been broadly categorized into the following areas: 1) Novel Concepts, Technology and Study Design; 2) Biomarker Discovery; 3) Biomarker Pre-validation; 4) Biomarker Validation; and 5) Team Science in Biomarker Development.

EDRN uses a set of metrics to evaluate the quality of the data resulting from supported projects, as well as their impact on the biomarker research field and the clinic:

- Completeness of data: Reproducible data on prioritization and down-selection of potential strong biomarker candidates.
- Quality of studies performed to date: Ability to retrieve previously established biomarkers provides confidence in approach.
- Transformative potential: Integration of multiple data types could improve success rate, knowledge content of biomarkers.
- Use in clinical setting: Pursuing the early detection of cancer with emphasis on aggressive disease to reduce the occurrence of overdiagnosis and overtreatment.
- Practice changer: It is quite possible that pathway-based selection of candidate biomarkers will improve their utility for early detection.
- Team science approach: Multi-center and multi-disciplinary effort to increase rigor and likelihood of project success.

Apart from the five early detection/diagnostic tests that have already been approved by the Food and Drug Administration (FDA) and the five that are currently being offered through Clinical Laboratory Improvement Amendments (CLIA) labs, some of the most commendable accomplishments of the EDRN during the past five years are highlighted below and also summarized in greater detail together with other scientific accomplishments in the following sections of this document:

- Harnessing Genomic Data to Guide Proteomic Analysis: Can Expression Profiling Identify Early Detection. Principal Investigator (PI): Michael Birrer, MGH. In an effort to identify new and better serum biomarkers for the early detection of ovarian cancer (OC), Dr. Birrer leveraged the information generated by extensive DNA/RNA and mutation/methylation data analyses of serous type OC along with the use of a secretome platform. This novel approach has led to the identification of known markers of OC, including CA125 and HE4, as well as to the discovery of new promising candidate biomarkers, such as FGF-18.

- TMPRSS2:ERG Fusion as Prostate Cancer Biomarker. PI: Scott Tomlins, University of Michigan. Until recently, PSA testing was the gold standard for screening for prostate cancer, and detection of its elevated expression triggered ~ 1,000,000 prostate biopsies each year. However, PSA has several well-known limitations as an early detection biomarker. EDRN investigators from the Chinnaiyan BDL identified fusion transcripts in ~50% of PSA-screened prostate cancers between TMPRSS2 and ERG genes. The fused transcripts are unique to prostate cancer and are not found in benign prostate tissue or in any other cancers. TMPRSS2:ERG fusions are found in HGPIN, which is considered the precursor lesion of prostate cancer. A Phase 3 randomized trial of men with HGPIN has shown that men with ERG+ HGPIN have a significantly higher risk of developing cancer than those with ERG- HGPIN. A urine-based early detection assay for TMPRSS2:ERG is already available at the Michigan U. CLIA-certified lab. It is expected to have a major impact by changing the clinical management of isolated HGPIN (~100,000 men/yr).

- Methylation Markers for the Detection of Colon Cancer. PI: Sanford Markowitz, Case Western University. The Markowitz BDL has discovered *Vimentin* gene methylation in stool DNA as a promising colon cancer biomarker. The marker is currently in a Phase 3 validation study in an asymptomatic screening population. If the performance of the biomarker observed in the Phase 2 study holds, its use in a clinical setting would be beneficial for patient compliance, detection of flat lesions in the right colon and/or the detection of occult upper GI neoplasms.

- The Airway Transcriptome as an Early Detection Biomarker for Lung Cancer. PI: Avrum Spira, Boston University. The Spira BDL addressed the area of field injury of irreversible changes related to lung cancer with an 80-gene expression signature (BronchoGen) from epithelial cell brushings obtained during bronchoscopy. This panel combined with bronchoscopy has progressed well from discovery to validation with a 95% and 93% NPV, respectively. BronchoGen is currently an approved CLIA test, which will be available in 2014. An FDA Phase 3 clinical trial involving 1,200 patients is also currently in process.

Introduction

The State of Biomarker Research: Before and After EDRN

Prior to the establishment of EDRN, biomarker research in the cancer community could be typified as lacking well-established guidelines for carrying out discovery, development, and confirmation of cancer biomarkers that meet a level of scrutiny for consideration for an FDA-approved diagnostic test. Common pitfalls that clouded the investigators' research in early detection or diagnostic cancer biomarker projects included performing studies that failed to recognize or minimize chance, bias and data overfitting, use of convenience samples that did not reflect the clinical context in which the biomarkers would be applied, and lack of using standardized operating procedures to meet stringent conditions and consistent collection of specimens from patients. The outcomes of studies performed with these oversights were results that could not be replicated in other laboratories or the disappointing inability to confirm the diagnostic performance of candidate biomarkers in independent specimens from different cohorts.

Investigators within EDRN early on sought remedies to these problems by establishing stringent guidelines for conducting biomarker research (1, 2). The guidelines developed by EDRN are now widely recognized by the research community as essential to increase the probability of success in biomarker discovery and development. For example, collection of specimens using the prospective-sample-collection, retrospective-blinded-evaluation (PRoBE) study design proposed by EDRN investigators offers a rigorous approach to eliminate much of the bias often encountered during all phases of biomarker discovery and validation. The need to confirm biomarker performance in samples collected independently from those used during discovery, and preferably coming from multiple sites, will establish whether the biomarkers demonstrate accurate discrimination or are merely the result of overfitting during the early phases of discovery. Statistically powered standard specimen reference sets have been assembled within EDRN to help the research community afford the samples needed to validate their biomarkers for their intended clinical use following the strict criteria mandated by the PRoBE study design. The involvement of multi-center participation in validation trials ensures that any new biomarker assays can be replicated at multiple institutions and that the test works in samples collected from a multitude of clinical sites. EDRN has also proposed a five-phase biomarker development strategy to guide the research community on advancing their biomarkers through defined steps to ultimately gain FDA approval and further testing in clinical settings. In conclusion, EDRN has been instrumental in changing the awareness of the biomarker research community by establishing rigorous standards by which biomarker discovery and validation are to be conducted and making high quality, statistically powered clinical specimen collections available that are too difficult to assemble by independent laboratories.

Biomarker Tests Promoted for FDA Approval or for Use in CLIA-Certified Laboratories

The progression of biomarkers from discovery to validation to FDA approval is a long and arduous task for academic researchers or companies. EDRN has made this process less cumbersome by creating and making available critical resources to facilitate validation of a number of biomarkers with the necessary rigor to achieve approval as diagnostic tools by the FDA. The following tests were all successfully approved by the FDA through the beneficial contributions of EDRN investigators during Phase 3 validation studies for each of the markers included in the tests: OVA1 (panel of 5 biomarkers) and Risk of Ovarian Malignancy Algorithm (ROMA) for differential diagnosis of malignant from non-malignant ovarian pelvic masses, proPSA and urine PCA3 tests for reduction of number of initial biopsy or rebiopsy for prostate cancer, and AFP-L3 and DCP tests for risk assessment for development of hepatocellular carcinoma. Additional markers are currently in the pipeline for which CLIA-certified tests have been established, as a result of close interactions among EDRN investigators, initial discoverers, and biotechnology companies. EDRN has instituted an environment of openness and collaboration to outside parties to facilitate promotion of promising biomarker candidates to achieve Phase 3 validation in CLIA-approved laboratories.

In addition to progressing effective biomarkers forward, EDRN-supported validation studies have served as a brake for other markers or technologies that did not demonstrate effectiveness in the early diagnosis of cancer. Microsatellite instability markers for recurrent bladder carcinoma, SELDI-based profiles for prostate cancer, and the OvaSure marker panel for ovarian cancer did not pass diagnostic scrutiny, thus halting further unfruitful efforts with these markers. The significance of these "failed" validation studies was that continued investment and interest in markers that offer no clinical benefit was terminated.

Biomarkers Discovered and Developed within EDRN

Discovery of early detection cancer biomarkers that will meet performance requirements to be of benefit in a clinical setting is the most challenging aspect of biomarker research. The collaborative environment of EDRN has improved the process to quickly weed out markers that will not meet these criteria, so that only those markers which continue to show promise following a series of rigorous prevalidation studies are promoted to move forward. Many biomarkers developed within or with the support of EDRN resources are currently in various stages of validation. The *TMPRSS2-ERG* gene fusion was a landmark discovery demonstrating for the first time gene fusions contributing to oncogenic progression in an epithelial cancer. This and other related gene fusions are now being studied in Phase 3 validation trials. Fibulin-3 was discovered and validated as a highly sensitive and specific marker of mesothelioma in either plasma or pleural effusion. GP73 was discovered as a marker for hepatocellular carcinoma with HCV etiology and is being used in a diagnostic setting in China. OVA1, a biomarker panel (CA 125, prealbumin, apolipoprotein A-1, beta2-microglobulin, and transferrin) that distinguishes ovarian malignancies from benign pelvic masses, was developed by EDRN investigators at the Johns Hopkins University Biomarker Reference Laboratory. This test represents the first ever in vitro diagnostic multivariate index assay (IVDMIA) cleared by the FDA. A second algorithm-

based test, ROMA (using CA125 and HE4 marker values), with the same clinical application as OVA1 was also recently approved by the FDA. This test was developed by EDRN investigators at the Massachusetts General Hospital Biomarker Discovery Laboratory. A current Phase 3 validation study on colorectal cancer includes two markers that have matured within EDRN and were selected following Phase 2 studies. A stool-based assay for methylation of the vimentin gene and a serum-based assay for galectin-3 ligand, a specific glycoform of haptoglobin, are included in this large, multi-year validation study.

EDRN investigators have also opened doors to many external partners to facilitate the progression of biomarkers through validation to ultimately approach FDA for approval. Such ongoing collaborations include those with Quest Diagnostics for a 10-serum protein biomarker panel for lung cancer, Ciz1 plasma marker for early stage detection of lung cancer, and a gene expression panel by Allegro Diagnostics for diagnosis of lung cancer in patients undergoing bronchoscopy. Aside from these promising leads, EDRN has also facilitated the elimination of many other markers that did not succeed in early prevalidation studies. This provides an important check for outside investigators alerting them to reallocate their efforts and resources on other promising markers.

Productivity of EDRN Investigators

The productivity of EDRN investigators is also reflected by other benchmarks commonly applied to most researchers. During its 13-year period, the EDRN investigators have published more than 1,900 peer-reviewed articles, of which approximately 22% are in high impact journals or have a citation index greater than 300. Many patents or licenses have been applied for revealing the practical applications sought within the Network; EDRN investigators currently have 64 patents and 12 licenses. Over 30 collaborations have been formed between EDRN laboratories and biotechnology or diagnostic companies seeking assistance in developing biomarkers. All of these activities have had a focused attention on more than 1,000 biomarkers that have been under consideration throughout the history of EDRN, among which approximately 300 have moved forward for consideration in prevalidation studies.

EDRN Informatics: An Infrastructure for Supporting Cancer Biomarker Research

Through collaboration with NASA, the EDRN has directly leveraged and worked with the Jet Propulsion Laboratory (JPL) to infuse and collaborate on the development of informatics technologies to support science-driven research on biomarker discovery and validation. In 2011, NASA presented a Group Achievement Award to this collaboration for innovative approaches and use of NASA software technologies to enhance biomarker research and link together biomarker research laboratories.

Through its interaction with JPL, the EDRN has developed a suite of informatics tools and services that support collaboration and data sharing of biomarker research results both within and outside the Network (http://cancer.jpl.nasa.gov/). This complete data management infrastructure

is equipped with the technology necessary to collect, store, and share the full spectrum of data generated from biomarker research:

- EDRN Biomarker Ontology — An information model with more than 2,000 Common Data Elements (CDEs) developed for use in biomarker research.
- EDRN Specimen System (ERNE) — A national infrastructure for locating information about biospecimens.
- EDRN Study Management System (eSIS) — A database of biomarker studies within the EDRN.
- Validation Study Information Management System (VSIMS) — A Laboratory Information Management System (LIMS)-based system for managing biomarker validation studies.
- Science Data Warehouse (eCAS) — An infrastructure for capturing, processing and distributing scientific data sets.
- Biomarker Database (eBMDB) — A database of annotated research results from the study of cancer biomarkers.
- Science Data Portal or EDRN Knowledge Environment (EKE) — A public portal infrastructure for accessing EDRN research results and information across the Network (hosted at the NCI: http://www.cancer.gov/edrn).

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Novel Concepts, Technology and Study Design

Overview

EDRN has built a vast array of enabling technologies to help discover and develop biomarkers and enrich the pipeline for further validation. The EDRN leadership ensures that there is a synergy among various technologies and the potential to integrate them for biomarker development clearly demonstrates the value of the EDRN in delivering a product that is greater than the sum of the individual projects. Integrated genomic and proteomic technologies are yielding a highly innovative strategy for identifying candidate biomarkers for early detection that draws upon the multiple disciplines represented within EDRN (i.e., clinical and basic science, technology development, biostatistics and bioinformatics). An efficient and cost effective way to rapidly verify potential candidate biomarkers developed by EDRN researchers and further refine a biomarker panel in pre-clinical validation studies is provided by employing highly sensitive targeted mass spectrometry-based technologies, such as SRM and PRISM-SRM, before further investment in the development of expensive, clinical-grade immunoassays. The Nucleic Acid-Programmable Protein Array (NAPPA) platform opens the possibility of exploiting the natural tumor-antigen signal amplification provided by autoantibodies to identify novel targets that could be used to develop more sensitive early detection biomarker assays. Some examples are highlighted to illustrate EDRN's integrated approach that simply could not have been achieved by a series of independent R01 grants. The fact EDRN investigators work together in the context of integrated workflows, such as those described in the representative studies below, is proof that the collaborative process within the Network is very effective.

Harnessing Genomic Data to Guide Proteomic Analysis: Can Expression Profiling Identify Early Detection

PI: Michael Birrer, M.D., Ph.D., Massachusetts General Hospital

In recent years, many hallmark advances have been made in the treatment of ovarian cancer; however, the rate of mortality remains flat and unchanged. A contributing aspect in the mortality rate may be the fact that the majority of ovarian cancer cases are detected in late stages. Predominantly, these late stage cancers initially respond well to therapy, but subsequently recur at a high rate. Thus, early detection of primary and recurrent ovarian cancer may be key factors in increasing overall survival and impacting mortality from the disease. To date, there are no FDA approved screening markers for ovarian cancer with the exception of only two markers for monitoring of recurrence. These markers, HE-4 and CA125 are problematic as they have poor sensitivity (only about 50% of stage I patients express elevated levels) and poor specificity (increased levels are detected in other gynecological disorders).

In an effort to identify new and better serum biomarkers, much work has been done in the genomics of ovarian cancer that includes extensive DNA/RNA analysis of serous type and mutation/methylation data analyses. These studies have yielded large amounts of genomic/epigenomic abnormalities that include areas of chromosomal gain and loss, hypermethylated genes, transcriptional cluster patterns, etc., but, surprisingly few high-frequency mutations.

The Birrer group is using a secretome-array platform approach to discover new serum markers. This secretome array construction has three phases: (1) Gather secreted protein/gene information; (2) Map to Affymetrix probe set; and (3) Assemble and remove redundancy. The proof of principle of this biomarker discovery approach was tested with ovarian patient samples. Two sets of data were put through the analysis: Dataset A: Cancer vs. Normal Fallopian Tube, and Dataset B: Cancer vs. Normal Ovarian Surface Epithelium. Both data sets were put together into the secretome platform and resulted in a number of probe sets. These probe sets were filtered in and out for pathway analysis and also for any markers that are highly expressed in other cancers or normal tissues. The results validated the ability of the secretome platform in identifying the two known ovarian cancer biomarkers (CA125 and HE-4), as well as other markers associated with ovarian cancer.

Further analysis yielded potential early detection/diagnostic markers, as well as potential novel therapeutic targets. One such discovered marker, FGF-18, has shown promise in distinguishing normal from cancer in ELISA assays.

In this study, the Birrer group created secretome array mapping probe sets that identify genes whose proteins are likely to be present in serum. Bioinformatic-based "validation" as well as results from initial experimental validation support the utility of the approach. The secretome platform may prove to be invaluable for the discovery phase of serum biomarkers and a helpful method to ensure that efforts and resources are used in validating "appropriate markers." The down-selection from over 1000 potential biomarkers, based solely on differential expression, to

17, based on genomic data, demonstrates the power of the genomics-based approach. The parallel mass spectrometry-based discovery effort is intriguing in that the protein candidates will apparently be down-selected using similar genomics-based criteria. The integration of multiple data types would likely improve the classification accuracy of the resulting biomarkers.

Integration of genomic, transcriptomic and proteomic data into a single unified picture of tumor biology, including identification of functional processes and pathways that differ between normal and tumor tissues is a transformation that has been a long time coming, but which is currently being pursued by a number of groups, within and outside of EDRN. If this approach is successful in generating a panel of biomarkers that achieve the necessary sensitivity and specificity for early detection of ovarian cancer, it would be transformative of clinical practice, as ovarian cancer is a disease which has seen no significant decrease in mortality over the past decade.

The clinical applications envisioned include both early detection and identification of new therapeutic targets. Pathway-based analysis is going to be crucial for identification of therapeutic targets, and it is quite possible that pathway-based selection of candidate biomarkers will also improve their utility for early detection. The specific experimental design, using both OSE and FTE as 'normal control' tissues is likely to contribute to our understanding of ovarian cancer biology and progression. The availability of a specific and sensitive blood-based test for early detection of ovarian cancer would have obvious and dramatic effects on clinical practice.

This project would not be possible but for the multi-disciplinary team effort involving clinicians, genomics and proteomics experts, biostatisticians and bioinformaticians. The strong preliminary data are an illustration of a well-constructed down-selection potential, with the identification of a strong candidate biomarker, FGF-18, and demonstrate the potential of integration of multiple data types to improve the success rate and knowledge content of biomarkers, as well as the possibility that pathway-based selection of candidate biomarkers may improve their utility for early detection.

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SRM-based Targeted Quantification for Candidate Biomarker Verification without Affinity Reagents

PI: David Camp, Ph.D., Pacific Northwest National Laboratory

The Pacific Northwest National Laboratory (PNNL) specializes in the development of improved mass spectrometry technologies for increasing the sensitivity and throughput of MS-based proteomics. Current efforts to improve the MS platforms for targeted proteomics measurements include improvements to existing triple-quadrupole MS platforms and the development of a penta-quadrupole MS.

Other approaches to improving the sensitivity of targeted MS measurements include subjecting the protein sample to a number of biochemical enrichment and fractionation steps, as the sensitivity of MS measurements is generally limited by the complexity and dynamic range of the sample being analyzed. PNNL has developed approaches based on immunoaffinity depletion of the most abundant plasma/serum proteins using IgY14 columns to remove the top 14 most abundant proteins and SuperMix columns for removal of moderately abundant proteins. Strong cation exchange liquid chromatography can also be used, in standard formats or in a new high pressure high resolution intelligent selection regime designated as PRISM-SRM.

In the context of the EDRN, PNNL was tasked with demonstrating the utility of MS-based proteomics as an alternative to the development and use of ELISA assays, particularly in conducting early verification and preclinical validation studies of candidate biomarkers derived from genomic and/or transcriptomic experiments. Initial proof-of-concept experiments were done using PSA as the target. It was discovered that the differential use of IgY14 and IgY14+SuperMix immunoaffinity depletion columns could separate total PSA (IgY14 flow through) from Free PSA (SuperMix flow through), in a manner analogous to current ELISAs, as the SuperMix columns remove alpha1 chymotrypsin, the major carrier protein for bound PSA.

In collaboration with the BRL at Johns Hopkins University (PI: Dan Chan), PNNL conducted a blinded study of 33 clinical serum samples of PSA comparing SRM to CLIA-approved ELISA assays. The PNNL SRM-MS assay matched the CLIA ELISA performance in analytical sensitivity and CV, and the correlation between the two different assays was better than 0.93.

PNNL then tested the limits of detection (LOD) and limits of quantification (LOQ) for various SRM strategies, using purified PSA spiked into female serum as the test system. The comparison is shown in the following table:

SRM-based assays at PNNL	LOQ
Conventional LC-SRM	~1 µg/mL
LG-SRM	~10 ng/mL
IgY14-LG-SRM	~1 ng/mL
PRISM-SRM	~1 ng/mL
IgY14-PRISM-SRM	~50 pg/mL

The PRISM technology is a two-stage process where the first stage is fractionation and then multiplexing followed by conventional SRM analysis. LG-SRM involves the use of a long LC gradient to increase throughput and decrease sample size requirements, at the cost of sensitivity. The second objective of the PNNL BRL was to demonstrate the utility of SRM-MS for detection of TMPRSS2:ERG fusions at the protein level, as well as the development of SRM assays that could distinguish between various potential T2:ERG isoforms. PNNL developed a set of three SRM assays that were able to distinguish between T2:ERG-positive and T2:ERG-negative cell lines and patient tissue samples with very good accuracy. Interestingly, two of the peptides represented mutually exclusive isoforms of T2:ERG. Observation of both peptides in the same tissue sample implied the presence of either multifocal disease or multiple splice variants from the same fusion gene, either one of which might have potential as a future prognostic indicator. This is a possibility that will be pursued in future studies. The quality of the data was further enhanced by the comparison with CLIA-approved ELISA assays for PSA, and with PCR and FISH-based assays for TMPRSS2:ERG fusion proteins.

PNNL is also working with the Breast/GYN Collaborative Group to develop SRM-MS assays for the rapid verification of candidate biomarkers for the early detection of ovarian cancer identified by various members of the Collaborative Group. PNNL was given a list of 14 candidate biomarkers lacking analytical antibodies, as well as CA125 and HE-4 as quality control targets. Using straight SRM-MS, PNNL was able to detect only 5 of the 16 biomarkers, but with PRISM-MS 14 of the 16 candidates were detected in a test sample of 5x5 ovarian cancer cases and benign pelvic mass controls provided by Nicole Urban. PNNL is currently analyzing a blinded set of 50x50 ovarian cancer cases and benign controls.

The ability of enhanced targeted mass spectrometry-based technologies to detect specific protein biomarkers at clinically relevant concentrations (less than 1 ng/mL in plasma or serum) is quite impressive. Depending on the requirements of the assay, technical approaches are employed that could either maximize sensitivity (better than 100 pg/mL) at the cost of throughput, or conserve sample size and increase throughput while still achieving low ng/mL sensitivity.

The transformative potential of the PRISM-SRM and LG-SRM technologies lies in the ability to significantly reduce the time and cost required to produce highly specific protein-based assays for verification of candidate biomarkers initially discovered from genomic or transcriptomic studies. The SRM and PRISM-SRM platform does not require target-specific antibodies (although it does benefit from the use of commercially available affinity reagents for immunodepletion of highly abundant proteins). The MS-based approach can thus obviate the need to generate peptide or protein-specific antibodies, and also provides a quality control check, peptide identification, and specificity of the assay. While other groups are also working on targeted MS-based assays, the PNNL group appears to have one of the best combinations of sensitivity and throughput.

The SRM and PRISM-SRM technologies are unlikely to have immediate direct clinical application, although the ability to distinguish among different isoforms, as in the case of T2:ERG fusion proteins, has a potential clinical application, should the different isoforms prove to have prognostic value. Nonetheless, the technology has significant potential to change the routine pipeline for biomarker verification and prevalidation, thus conserving resources for a

smaller set of more stringently tested candidate biomarkers that can be developed for ELISA-based tests of highly valuable samples.

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Self-Assembling Protein Microarrays for the Discovery of Autoantibodies

PI: Joshua LaBaer, M.D., Ph.D., Arizona State University

For breast cancer detection, the current diagnostic modalities involve imaging by mammography and MRI. Neither of these tests have 100% sensitivity and specificity for diagnosis. Clinicians and cancer researchers alike have been searching for biomarkers to augment the currently available imaging tests.

Based on the idea that cancer patients spontaneously produce antibodies against "tumor antigens", Dr. LaBaer's group has developed the Nucleic Acid-Programmable Protein Array (NAPPA) technology for detection of autoantibodies. The latter are advantageous in that they often predate the presentation of cancer and they persist long after the triggering antigen disappears (sometimes for decades). Often, too, they can amplify the cancer "signal" and are very stable in serum samples. While the use of autoantibodies as biomarkers is not revolutionary, the NAPPA-based approach is an extremely powerful way to screen and evaluate autoantibodies on a proteome-wide scale and with a throughput that generates data from enough patients for meaningful statistical analysis. Although in its array format it can be successfully used to identify and validate new biomarkers, it is not portable for wide-spread clinical use. However, the NAPPA technology can be modified for that purpose into a 96-well plate format or could ultimately be converted into a more conventional ELISA assay. NAPPA is an excellent approach for protein-based personalized diagnostics for high risk populations and could be used to supplement screening for basal-like breast cancer using mammography and/or MRI technologies.

The NAPPA technology improves conventional protein arrays by printing the genes that are responsible for a specific protein, rather than directly printing the peptides or proteins. In this way, the proteins are synthesized "in situ", which results in proper anchoring of biologically active proteins.

Recently, the LaBaer group has made further improvements on the technology. The antigen pool is now greater than 10,000 human proteins; expressed with the use of human ribosomes derived from HeLa-cell lysates for better protein yield and native folding; much of the work is now done by robotics; and non-specific background has lessened with the use of *E. coli* lysate blocking procedures.

Although there are many advantages to using autoantibodies as biomarkers for cancer, one disadvantage is that there is great heterogeneity within the population in terms of antibody expression. For example, some people do not express increased levels of antibodies against accumulated p53 regardless of cancer stage, while other markers, like EBNA-1, may be highly expressed in normal as well as in cancer patients.

In order to lessen the confounding factors of heterogeneity of expression, a case-control study was performed using a subset of breast cancer patients with very specific clinical criteria. The study used 148 patients with basal-like breast cancer (triple negative) with the added characteristics of EGFR+ and CK5/6+ where good molecular profiling data was available. The

study has three phases: discovery, prevalidation/training and blinded validation. Discovery and prevalidation results revealed six promising markers with reasonable sensitivity and specificity. Future plans are to perform a blinded validation study.

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Biomarker Discovery

Overview

The process of biomarker development from discovery to clinical implementation in multiple biocompartments is only possible due to collaborations among cross-disciplinary clinical and laboratory researchers at multiple institutions, collaborations with industry, and access to high-quality, well-annotated, uniformly collected specimens through the EDRN. This is illustrated in the ground-breaking discovery by EDRN BDL investigators of the TMPRSS2 and ERG gene fusion as a unique biomarker for prostate cancer. With industry collaboration, the investigators developed a quantitative assay for TMPRSS2:ERG quantification in urine. In addition, a urine-based early detection CLIA-certified assay for TMPRSS2:ERG is available at the University of Michigan. In case of pancreatic cancer, the discriminatory ability of a combined panel of MUC4, MUC5AC and CA19-9 to distinguish pancreatic cancer from healthy controls was tested compared with CA19-9 alone. Another example is the discovery of methylated *Vimentin* gene by EDRN BDL investigators as a colon cancer biomarker. An assay was developed, standardized, and successfully transitioned to an EDRN BRL. Currently, EDRN is conducting a large, multi-center, prospective validation study to test this biomarker in stool from an asymptomatic population, along with other serum markers for the early detection of advanced adenomas, high grade dysplasia and colorectal cancer. EDRN investigators are also examining the release of tumor DNA (tDNA) into either stool or blood for possible identification of cancer biomarkers. The use of tDNA has the potential to improve detection of cancer by focusing on specific DNA changes. This will be dependent on the DNA selected for the specific cancer and also on the technology used to detect the tDNA. There are technical challenges for detecting tDNA, which are being addressed by the investigators. The studies performed show proof of principle for detection of tDNA having the potential to detect a multitude of different cancers, including CRC, ovarian and endometrial cancers. This approach also affords the opportunity to examine and detect the emergence of resistance mutations that develop as a consequence of chemotherapy.

TMPRSS2:ERG Fusion as Prostate Cancer Biomarker

PI: Scott Tomlins, MD, PhD, University of Michigan

The TMPRSS2:ERG biomarker has gone through the entire process from discovery through assay development and prevalidation, and is currently being validated by the EDRN GU Collaborative Group. Until recently, PSA testing was the widely used method of screening for prostate cancer. PSA is a tissue marker and not a cancer marker and has several limitations as an early detection biomarker. Detection of elevated PSA has triggered ~1,000,000 prostate biopsies each year. As reported by the Prostate Cancer Prevention Trial (PCPT), PSA also has sensitivity limitations, where 15% of men with PSA levels of 0 to 4.0 ng/mL had prostate cancer of whom, 15% had high Gleason grade disease. The phenomenon of overdiagnosis of prostate cancer is mostly attributable to PSA screening: it is thought that 23-43% of all screen-detected cancers would never have caused symptoms, thus indicating overtreatment. Recently, EDRN investigators from Arul Chinnaiyan's research team identified fusion transcripts between TMPRSS2 and ERG genes in ~50% of PSA-screened prostate cancers. The fused transcripts are unique to prostate cancer and although they are not present in benign prostate tissue they are found in HGPIN, which is considered the precursor lesion of prostate cancer. The laboratory has successfully developed monoclonal antibodies to detect by IHC the truncated ERG protein, which is only expressed in cancers harboring the TMPRSS2:ERG fusion transcripts. ERG protein expression was also evaluated by IHC in a phase 3 randomized trial of men with HGPIN and was shown that men with ERG+ HGPIN have a significantly higher risk of developing cancer than those with ERG- HGPIN. ERG IHC test is now used clinically in challenging diagnostic cases and a urine-based early detection assay for TMPRSS2:ERG fusion is also now available in a CLIA-certified lab at the University of Michigan. It is expected that publication of the results of the phase 3 trial of HGPIN will have an immediate impact and will change clinical management of isolated HGPIN (~100,000 men/yr). Collaborative efforts of the two laboratories of Arul Chinnaiyan and Mark Rubin have led to the development of a molecular assay as well as an IHC assay, both of which are useful for the analysis of biopsy and prostatectomy specimens; in addition, they have developed a urine-based assay for non-invasive detection of the biomarker.

Through collaboration with industry, the investigators have developed a quantitative assay for TMPRSS2:ERG, detectable in whole urine following a digital rectal examination. Across >3,000 samples, urine TMPRSS2:ERG combined with PCA3, a non-coding RNA, shows significant improvement over serum PSA for predicting the presence of cancer upon biopsy. An EDRN validation study of urine TMPRSS2:ERG combined with PCA3 in pre-biopsy urine is currently ongoing. Most importantly, urine TMPRSS2:ERG is strongly correlated with total ERG tumor burden in a given patient. Preliminary studies suggest potential utility in risk stratification.

As part of this process, a validation study for PCA3 was conducted in a collaboration between an EDRN BRL (Dan Chan) and GenProbe, and coordinated by the EDRN DMCC. PCA3 was recently approved by the FDA.

EDRN investigators have also developed an assay to improve the sensitivity of PSA and the Prostate Cancer Prevention Trial (PCPT) Prostate Cancer Risk Calculator (PCPTRC) combined index. TMPRSS2:ERG fusion biomarker, as well as biomarkers more recently discovered by the

same investigators, such as mutant genes (e.g., SPOP) or overexpressed genes (e.g. SPINK1) provide a full coverage of TMPRSS2:ERG-negative prostate cancer and has improved the detection, diagnosis and stratification of prostate cancer. In addition, combinations of markers, such as TMPRSS2:ERG and PCA3, have a superior performance in detection and diagnosis of prostate cancers as compared to PSA isoforms or the PCPT index. The biomarker discovery, development, verification and validation were and are being conducted by a multidisciplinary team of researchers including molecular biologists, bioinformaticians, statisticians, urologists, pathologists, assay developers and an industrial partner that built a robust assay for PCA3, which is used in this study as well.

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Improving the Diagnosis of Pancreatic Cancer: A Combination of MUC4, MUC5AC and CA19-9

PI: Surinder Batra, PhD, University of Nebraska Medical Center

The EDRN BDL at the University of Nebraska (Dr. Batra) conducted a study to test MUC4 and MUC5AC as biomarkers for pancreatic cancer, and to utilize these markers as a panel to improve the performance of CA19-9. The results demonstrated upregulation of the markers in PanIN lesions, with MUC4 showing 100% specificity for differentiating adenocarcinoma from benign diseases in FNA samples. Efforts were also directed toward development of a SERS platform for the detection of MUC4 in serum, in which MUC4 differentiates early pancreatic cancer (stage I and II) from healthy controls with a sensitivity of 63% at 95% specificity. The platform also differentiated early stage cases from chronic pancreatitis with the combination of MUC4 and CA19-9, thus further improving this performance. In addition, MUC5AC was examined in a separate set of samples. MUC5AC ELISA assays differentiated pancreatic cancer from healthy controls with 71.2% sensitivity and 87.3% specificity. A combination of MUC5AC and CA19-9 further increased the performance of the individual markers, demonstrating 75% sensitivity and 81% specificity in differentiating early pancreatic cancer from healthy controls, and 86% sensitivity and 74% specificity in differentiating early disease from chronic pancreatitis. Finally, preliminary results using a limited set of samples showed that the combined panel of MUC4, MUC5AC and CA19-9 increased significantly the predictive value of CA19-9 alone to distinguish pancreatic cancer from healthy controls to a sensitivity of 95% and specificity of 100%. Overall, this panel is considered to have potential as an early diagnostic marker test for pancreatic cancer.

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Methylation Markers for the Detection of Colon Cancer

PI: Sanford Markowitz, MD, PhD, Case Western Reserve University

The Markowitz BDL has identified *Vimentin* gene methylation as a colon cancer biomarker. In a case-control prevalidation study, the sensitivity of methylated vimentin in stool DNA (sDNA) for stage I/II colon cancer was 84% (86% for adenomas >1 cm), at a specificity of 83-90%. The next step is the testing of methylated vimentin in sDNA in an asymptomatic screening population. Along these lines, EDRN has launched a validation study for the early detection of high-grade dysplasia, colon cancer and advanced adenomas to test the performance of methylated vimentin in sDNA, FIT, and other markers (galectin-3 ligand in serum). This will be done using samples collected prospectively at multiple academic clinical centers and by NCI's CCOPS Program, tested at an EDRN BRL (Dr. Stass). The Markowitz group has developed a standardized quantitative real-time MS-PCR version of the vimentin endpoint MSP sDNA assay. This assay was successfully transferred to the EDRN BRL in a CLIA-compliant format. The testing will be performed on blinded samples and the data will be analyzed by the EDRN DMCC. This study has progressed through the following phases of biomarker development: Phase 1, initial discovery of the methylated vimentin; Phase 2, detection of methylated vimentin in stool of patients with colon cancer (stages I and II); and Phase 3, validation of the performance of methylated vimentin in sDNA in an asymptomatic screening population. The PI of the Phase 3 multi-center validation study (GLNE-10) is Dr. Brenner.

The Markowitz group is also examining the implications of a positive stool methylated vimentin DNA test and a negative colonoscopy, i.e., a "false positive" test. Does this false-positive test indicate a missed flat lesion in the right colon, early diagnosis of a molecular clonal expansion or an upper GI neoplasm? To address this, Dr. Markowitz in collaboration with the EDRN GLNE-10 and the Case Western GI SPORE have launched a longitudinal follow-up of patients with "false positive" sDNA tests. After one year, a repeat of the stool methylated vimentin DNA test, colonoscopy and upper GI endoscopy will be performed to determine if the positive methylated vimentin test detected colon cancer or other GI cancer or if it was associated with a "false positive" sDNA test.

The use of methylated vimentin has the potential of changing the practice of colon cancer screening. Along these lines, Dr. Markowitz is also developing a circulating methylated vimentin DNA test as a biomarker. The sensitivity of the latter is lower than in stool (52% for stage I and II colon cancer), but its specificity remains high - 95%. The group is attempting to improve the sensitivity of the test by switching from a methyl-BEAMing technology to a NextGen sequencing-based assay.

Dr. Markowitz has also shown that methylated vimentin was detected in Barrett's esophagus, high grade dysplasia, esophageal adenocarcinoma, and squamous cell carcinoma. This has the potential for being the first biomarker for non-endoscopic surveillance of esophageal neoplasia (>90% sensitive).

The use of methylated DNA as biomarker test(s) has transformative potential. Dr. Markowitz has indicated that there are potentially additional methylated DNA loci that remain to be tested.

Early studies show that CpGs that become methylated in tumors are defined by 205 high stringency patches of DNA. Validation of these patches is currently underway using new colon cancer samples to determine their sensitivity and specificity. Dr. Markowitz is also working with an industrial partner, Exact Sciences, to develop the commercial aspects of his methylated vimentin biomarker. Stool DNA samples from the EDRN validation study along with blood from post-operative patients in the Case Western GI SPORE study will be used to also compare the performance of the circulating methylated vimentin to CEA in a prospective longitudinal study of post-operative colon cancer patients.

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Cell Free Tumor DNA as a Clinical Biomarker

PI: Ken Kinzler, PhD, Johns Hopkins University, School of Medicine

The Kinzler's BDL group is looking at the release of tumor DNA (tDNA) as a biomarker for colon cancer. Tumor DNA can be detected in either stool (stDNA) or circulating in blood (ctDNA). The advantage of this approach is the detection of small intragenic somatic mutations, methylated DNA or translocations/rearrangements.

Dr. Kinzler's group addresses the technical challenges of detecting tDNA by using BEAMing, NextGen sequencing, and finally by improving on sequencing by tagging starting molecules with endogenous or exogenous unique identifiers (UIDs). This Safe-SeqS strategy can decrease the error rates of sequencing for the detection of tDNA. Using Safe-SeqS technology, a survey of ctDNA in human cancers was performed. Stage IV of various tumor types (e.g., breast, colorectal, endometrial, ovarian, hepatocellular, etc.) were examined for ctDNA. The highest frequencies of detection of ctDNA (up to 100%), were found in the ovarian, colorectal, bladder and gastroesophageal cancers. All cancers examined showed some level of ctDNA. Detection of ctDNA ranged from 48-73% in localized disease and from 84-100% in metastatic disease. Both point mutations and rearrangements were found.

In metastatic colorectal cancers, codons 12 and 13 of KRAS were sequenced in a blinded study. Plasma from 127 of 128 KRAS wild-type patients was negative for mutations resulting in a specificity of 99.2%. Plasma from 68 of 78 KRAS mutant patients was positive with 100% concordance for the specific base change and a sensitivity of 87.2%. An important aspect of detection is the source of ctDNA. In localized colorectal cancer, 91% of cases had detectable ctDNA in stool and 61% in plasma. Similarly, when ctDNA was assessed in earlier cancer stages, the frequency of detectable cases of ctDNA dropped from over 80% to approximately 40%. The detection of ctDNA was much lower in adenomas - 75% in stool, and only 10% in plasma.

ctDNA can also be used to monitor therapy and discern the emergence of resistance mutations. Known resistance mutations in KRAS, BRAF, EGFR and PIK3CA were examined in cases of metastatic CRC who had initially responded to EGFR blockade but then progressed. A detectable resistance mutation was present in the plasma of 27 of the 28 cases included in the study.

Dr. Kinzler's group also demonstrated the successful detection of tDNA from PAP smear specimens with a 100% sensitivity for endometrial cancer, and 41% sensitivity for ovarian cancer. A 12-gene panel was used which was estimated to detect >90% of all mutations in both endometrial and ovarian cancers.

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Biomarker Prevalidation

Overview

Prevalidation is paramount to the transition of biomarkers from the discovery/development phase toward testing on independent, blinded samples to determine if the biomarkers should progress to more extensive studies carried out on a larger scale and in a clinical trial context. The scientific literature is inundated with biomarkers at initial discovery stages with claims of explicit association with various types of cancers, yet these biomarkers often are not found to progress further towards clinical application. There are multiple reasons for failure to progress to the next phase of development. EDRN has facilitated this process through establishing recognized guidelines on the basis of which biomarkers should be evaluated as to whether their performance merits further consideration for larger scale clinical validation trials. Prevalidation constitutes the evaluation of biomarkers using samples distinct from those used in the discovery or early development stages, preferably collected from multiple sites and in compliance with the PRoBE study design criteria. The testing lab must be blinded to the status of the specimens to effectively measure the reliability of the biomarker test for its intended use in a clinical setting. Furthermore, the prevalidation study must be sufficiently powered to permit reasonable assessment of biomarker performance. The prevalidation study is intended to serve in evaluating whether a biomarker achieves an acceptable performance in a clinically defined disease scenario to warrant further development in more costly clinical validation trials. A positive result from a prevalidation study is clearly desired, but even failed tests can offer benefit in demonstrating that further development of certain biomarkers should no longer be pursued.

The two representative studies described below highlight different aspects of prevalidation used in the EDRN. In the study on the early detection of lung cancer, Dr. Avi Spira developed a transcriptomic signature from bronchial brushings as a diagnostic test for lung cancer. This test is based on the recognized "field effect" and has since progressed to a full clinical validation study. It is expected to be available as a CLIA test in 2014, with FDA approval being considered shortly thereafter. Although much of this work was initiated before Dr. Spira joined the EDRN as a PI, he always points out how instrumental EDRN had been in helping move this research forward, through his many interactions with EDRN investigators during the earlier prevalidation phases of the test. As a current EDRN investigator, Dr. Spira is expanding the applicability of the field effect gene expression signature to nasal epithelium, a more readily accessible site that could be more amenable to assessment of indeterminate nodules detected by CT. In the highlighted study on breast cancer, 90 biomarkers found in the literature were tested on invasive and localized breast cancer against benign breast disease by Dr. Jeffrey Marks in collaboration with investigators at MesoScale Diagnostics. The tested samples were obtained from the recently assembled EDRN standard breast reference set. Although the prevalidation results failed to reveal any biomarkers with an acceptable performance to discriminate cancer from benign disease, this study was quite conclusive in eliminating all 90 markers from further consideration by the research community. Without the resources made available by the EDRN for this prevalidation, other laboratories would likely continue their futile efforts to study many of these biomarkers.

Another study worth mentioning is Dr. Harvey Pass's discovery and prevalidation of fibulin 3 as a biomarker for malignant mesothelioma. Because mesothelioma is not a common disease, specimens for testing are difficult to obtain. Dr. Pass was able to collaborate with investigators at the Princess Margaret Cancer Center in Toronto who had an independent collection of plasma from mesothelioma cases and from appropriate asbestos-exposed controls. This initial prevalidation study proved promising as the performance of fibulin 3 in the Toronto samples (AUC=0.87) was nearly as good as that demonstrated with his own cohort used for the discovery of the marker (AUC=0.90). Additional validation is currently being planned with another cohort in Chile.

The Airway Transcriptome as an Early Detection Biomarker for Lung Cancer

PI: Avrum Spira, Boston University

Dr. Spira's research addresses the area of field injury of irreversible changes related to lung cancer with an 80-gene expression signature (called BronchoGen) from epithelial cell brushings obtained during bronchoscopy. This gene signature combined with bronchoscopy has progressed well from discovery to validation with a 95% and 93% NPV, respectively. A higher false-positive rate associated with severe COPD is apparently due to inflammatory responses, which may confound the current panel. Current progress includes a CLIA approved test (BronchoGen), which will be available in 2014. An FDA phase 3 clinical trial involving 1,200 patients is now in progress.

Recent developments along this vein include the evaluation of specimens obtained from nasal epithelium for their potential as an effective and less invasive source of RNA also encompassing the field effect of smoking injury. Analysis of smoking-induced gene expression changes in nasal epithelium that may mirror those found in the bronchus identified a 5-gene signature associated with lung cancer development. Prevalidation of this 5-gene panel demonstrated the ability to discriminate and predict lung cancer, but with lower performance than the BronchoGen test. Additional efforts to improve performance include the incorporation of miRNAs into the nasal signature.

BronchoGen data addresses the ability to assess indeterminate nodules detected by CT or enhance the results of bronchoscopy. The data provides the ability to distinguish stage I lung cancer from benign conditions.

The progress and success of this research is impressive. To have a CLIA test ready in 2014 and the potential for FDA approval in the very near future demonstrates how well this group has transitioned from discovery to prevalidation to validation. For the FDA to take interest in BronchoGen clearly indicates the prevalidation studies have met its rigorous criteria.

The tests developed by Dr. Spira could provide the ability to change current clinical practice in assessing those individuals at high risk for lung cancer. The BronchoGen test is currently being assessed for suitability for Medicare reimbursement. While the test has the potential of sparing some patients from an invasive procedure, it also helps the clinical community through its cost effectiveness.

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Prevalidation of Biomarkers for Invasive Breast Cancer

PI: Jeffrey Marks, Duke University

Dr. Jeffrey Marks and his collaborators at MesoScale Diagnostics conducted a prevalidation study in which a set of 90 candidate biomarkers, many of which were identified from the literature, was tested for its ability to distinguish invasive and/or localized breast cancer from benign controls. The investigators used a standard breast reference set which was recently assembled by four EDRN collecting sites based on established SOPs and according to PRoBE study design criteria. The markers were tested on 505 samples, divided into training and validation sets, in a blinded and randomized fashion using the MesoScale proprietary detection platform, and data was analyzed by the DMCC. The results demonstrated that none of the markers or any combination of them achieved a significant performance in distinguishing cancer from benign. At the same time, a panel of five biomarkers demonstrated discrimination between healthy controls from benign conditions or cancer. Importantly, samples from healthy controls included in the study were obtained from screening mammography clinic, while the cancer or benign disease samples were obtained from the diagnostic radiology clinic. It is therefore likely that lack of compliance with the PRoBE study design among the two types of samples led to the observed differences, thus, attributed to bias in the sample collection protocol and not due to biological differences. Among the 90 tested markers, only CA125 (MUC16) was found to be elevated (AUC=0.7) in cancer as compared to benign controls; however, this finding involved only the subset of women with ER-negative breast cancer. This finding is in line with the observed biological similarity between basal-type breast cancers and serous ovarian cancers.

The results of this collaborative effort helped preclude further consideration of the reported 90 biomarkers for the early detection of breast cancer. The study highlighted how EDRN is educating the scientific community to avoid such ill-fated studies during discovery or prevalidation and to avoid futile research on candidate markers that have now been proven ineffective. It is also a demonstration that a definitive and exhaustive study of this magnitude would be difficult to perform without the resources generated under stringent SOPs and collaborative efforts afforded by the EDRN. The inclusion of healthy controls obtained under different conditions is also a demonstration that failure to strictly follow PRoBE study design criteria can lead to false-positive results due to unforeseen inherent experimental bias.

Another EDRN study examining epidemiological risk factors for ovarian cancer using a multicenter cohort with 5,669 controls and 9,452 ovarian cancer cases demonstrated that the addition of a panel of 11 polymorphic SNPs associated with ovarian cancer had negligible effect on the predictive performance of the risk factors by increasing the AUC from 0.633 to 0.645.

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Biomarker Validation

Overview

The two projects discussed in this section illustrate the significance of EDRN's unique infrastructure and its expertise in long-standing support for biomarker development for early cancer detection as well as its recent emphasis on providing diagnostic and prognostic biomarkers, which enables better utilization of our limited national and global healthcare resources. The projects are of high quality, have major transformative potential and the ability to quickly impact clinical care. Both projects have also created unique biospecimen repositories, which can serve as a valuable tool for the rapid evaluation of other potential biomarkers.

The project on validation of biomarkers for colorectal cancer (PI: Dean Brenner) is the most comprehensive example of what the EDRN can accomplish by involving all components of the EDRN (BDLs, CVCs, BRLs and DMCC) and industry partners. If the proposed biomarkers perform as projected, there is potential to replace colonoscopy and fecal-based screening tests as first-line screening tests and reserve the performance of colonoscopy to an enriched subset of patients at high risk for having an advanced adenoma, high grade dysplasia or colorectal cancer. The prostate cancer project is also of high significance. Unlike the project on colorectal cancer, which focuses on biomarkers for screening, the Canary Prostate Active Surveillance Study (PI: James Brooks) is aimed at reducing the rate of overtreatment of prostate cancer.

The highlighted studies demonstrate the EDRN's ability to support projects with great promise to change clinical practice that would not be funded by conventional grant mechanisms. By using EDRN's expertise in biomarker development, study design, and biospecimen collection, along with its collaborative environment internally and with non-EDRN academic centers and industry partners, these projects will have the capacity to not only validate current biomarkers, but also evaluate the performance of new promising biomarkers.

Validation and Comparison of Biomarkers for the Early Detection of Colorectal Adenocarcinoma

PI: Dean Brenner, M.D., University of Michigan

Dr. Brenner and his co-investigators are conducting a large cross-sectional, EDRN PRoBE-compliant validation trial of stool-based and serum-based biomarkers for the detection of colorectal neoplasia. They proposed collecting blood, stool and urine from 4,800 subjects prior to a screening or surveillance colonoscopy (6,000 enrolled, assuming 20% drop out rate) to yield 72 cases (colorectal cancer or high grade dysplasia). To date, 3,900 subjects have been evaluated. The current rate of accrual is 50 subjects per week and enrollment should be completed by the spring of 2014. Both academic and community center sites are accruing subjects for this trial. The community sites are from the NCI's Community Clinical Oncology Program (CCOP).

The biomarkers to be validated in this trial are methylated vimentin in stool, galectin-3 ligand in blood, and EXACT Science's DNA panel in stool. Methylated vimentin was developed by Sanford Markowitz, PI of an EDRN BDL, and galectin-3 ligand was developed by Robert Bresalier's laboratory, which is part of Dr. Brenner's EDRN CVC. In addition to testing their DNA panel, EXACT Science has provided all of the stool collection kits for this trial. All three biomarkers included in the trial have been shown in case-control studies to have better sensitivity and specificity than FOBT and FIT. The assay for methylated vimentin has been successfully transferred at a CLIA grade to Dr. Sanford Stass' EDRN BRL, and the assay for galectin-3 ligand has been transferred to Dr. David Chia's EDRN BRL.

To date, case samples were collected from seven subjects with cancer, from 16 with high grade dysplasia, and from 549 with advanced adenomas. To increase the yield of cancers and high grade dysplasia, Dr. Brenner has altered the inclusion criteria to exclude subjects younger than 60 years and those with a prior colonoscopy within 108 months; there are already more than enough samples to power the study for the detection of advanced adenomas.

Stool, serum, plasma and urine collected for this trial will be available to validate additional biomarkers discovered by both EDRN and non-EDRN investigators.

This well-designed and conducted trial addresses a significant clinical problem and involves all components of the EDRN (CVC, BDLs, BRLs and DMCC), as well as CCOP accrual sites and an industrial partner. The investigators have accrued strong preliminary data on all of the biomarkers to be tested. The only area of concern has been the low yield of cancers and high grade dysplasias, which is being addressed by the change in eligibility criteria. Even if they do not reach the originally projected numbers, there will be a sufficient number of advanced adenomas to determine the ability of these biomarkers to detect the presence of such lesions, which is the secondary endpoint of the study. Most gastroenterologists believe that it is important that a screen test detects advanced adenomas as well as cancers and high grade dysplasias. This study has the potential to both reduce unnecessary colonoscopies and encourage those that need a colonoscopy to do so. The biomarkers are also likely to increase the detection of right-sided colon cancers which are poorly detected by colonoscopy. If these biomarkers

perform as projected, they may replace the use of FIT and colonoscopy for routine screening for colorectal cancer.

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Canary Active Surveillance Project (PASS)

PI: James Brooks, M.D., Stanford University Medical Center

Active Surveillance (monitoring of cancer with selective intervention upon indication of progression) is increasingly becoming the accepted management strategy for low risk prostate cancer. The Prostate Active Surveillance Study (PASS) design was formulated with the idea of having flexible and broad criteria. The study is open to all men with localized prostate cancer and collects full clinical information, dietary history, quality of life factors, psychometric measures, medications, etc. Disease progression was defined as an increase in Gleason score, volume of greater than 33% of biopsy cores, and/or clinical progression.

The PASS Study was opened in 2008 with the goal of recruiting 1000 patients. There are nine centers accruing specimens with DMCC oversight (study design and data analysis for all projects, online data entry, monitoring compliance with the protocol, statistical analysis, and compliance with data safety monitoring board). The goal is to collect three-year follow up data on all patients. The biospecimens collected are blood, urine, buffy coat and FFPE samples, which are shipped to a common biorepository. These specimens will be made available to any investigators with promising preliminary data.

To date, there are nearly 900 patients already on protocol with follow up data for some, albeit a short follow up. An increase in grade was the most frequent determinant in "disease progression." Sixteen percent of the current cohort has been treated, and one-third of those who chose treatment have not progressed. The study is ongoing with evaluation at each interval for PSA velocity and clinical markers of progression, temporal changes in urinary PCA3, and TMPRSS2-ERG analysis in conjunction with Gen-Probe, and with Genomic Health, a study of gene expression profiles that may predict disease progression.

The Canary Foundation is also participating in this study by creating a Tissue MicroArray (TMA) repository, which will be used to validate up to 75 biomarkers for prostate cancer progression. The TMA is designed as a case-control study from prostate tissues from a radical prostatectomy cohort with a five-plus year follow up. So far, there are 1,200 cases with four core biopsies per case. Seven-hundred of the cases in this cohort have no recurrence. Several biomarkers from various investigators are already being evaluated and they include: ERG, SPINK, PTEN (FISH and IHC), reactive stroma, image analysis, p27, Ki67, and MUC1.

PASS is an invaluable resource for future studies as it has collected many data elements (quality of life, dietary and medicine information) in its questionnaires; it serves as a platform for biomarker validation studies; and has spawned funding for additional and future studies. Unlike other active surveillance studies that only enroll low risk patients, PASS enrolls both low- and high-risk patients. These broader enrolment criteria reflect more accurately the situation faced by clinicians in their practices.

The PASS study directly addresses the issue of overdiagnosis of prostate cancer and can answer questions such as:
- What is the optimal follow up time for patients?

- Should patients be biopsied more or less frequently?
- What is the natural history of low grade prostate cancer?

If biomarkers tested in these specimens can accurately predict progression of prostate cancer, they can be used by physicians to advise patients on whether they should have a prostatectomy or consider enrollment in active surveillance. This is also a good example of team science as it involves the participation of several EDRN CVCs and the EDRN DMCC. The CANARY foundation has supplied funding to the accrual sites and the EDRN has funded the study coordination by the DMCC. Recently, industry has come forward to join the study. NCI's reputation enabled willingness for others to collaborate and the study used EDRN SOPs for specimen collection and quality assurance.

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Team Science

Overview

This section focuses on five representative Team Science projects – multidisciplinary collaborative projects which involve several laboratories from each of the EDRN Collaborative Groups. Two projects are from the GU Collaborative Group, one from the Lung Collaborative Group, one from the GI Collaborative Group, and one from the Breast/GYN Collaborative Group.

Biomarkers of Risk for Colorectal Cancer

Leading PI: Robert Schoen, M.D., M.P.H., University of Pittsburgh

The objective of the EDRN team project led by Dr. Schoen is to develop better modalities for post-polypectomy and post-CRC resection surveillance. This is a major clinical problem in that:
1. Surveillance colonoscopy is equivalent to average risk screening and therefore represents a major public health issue and expense; and
2. The yield of surveillance colonoscopy for screen relevant neoplasia (advanced adenomas and early stage cancers) is low (generally <10-15%). Thus, in retrospect, the majority of tests are wasted. Risk stratification of patients would be of major importance and the best use of biomarkers.

The investigators will use a variety of biomarkers including:
1. Bill Grady: U. of Washington/FHCRC – methylated genes
2. Paul Lampe: FHCRC – antibody arrays
3. Dan Liebler: Vanderbilt – proteomic analyses
4. Sandy Markowitz: Case Western – 15PGDH
5. Ken Kinzler: Johns Hopkins - detection and quantification of non-clonal somatic mutations

The investigators plan to:
1. Characterize inter- or intra-subject reproducibility and variability—this part has been largely accomplished with a rigorous demonstration. The data is compelling, showing good reproducibility between biopsies in the area, as well as changes in methylation patterns with age.
2. Characterize inter- or intra-subject variability by adenoma phenotype (normal vs. adenoma), which remains to be accomplished.
3. Evaluate biomarker expression in relation to long term adenoma recurrence, which remains to be accomplished.

If successful, this could be a practice changer since it would offer more rational decisions regarding surveillance intervals. This is important given the issue of both overuse of

colonoscopy and interval cancers (cancers occurring between colonoscopies largely from missed lesions).

Lung Team Project

Leading PI: Pierre Massion, M.D., Vanderbilt-Ingram Cancer Center, Vanderbilt University

A primary aim of the Lung Team Project 2 (LTP2) is to recruit 200 subjects with indeterminate nodules for bronchoscopy and blood draw. The inclusion criteria have recently been broadened to age >45 yr, smoking >20 pack-yr, nodule size 7-30 mm in linear direction, and no previous cancer. DMCC sample size calculations are for n=200 with 30 lung cancers and the remainders - controls with benign lesions. Most likely, a greater proportion of lung cancers will be recruited. There are four sites recruiting patients: NYU School of Medicine, Boston U, UCLA, and Vanderbilt. At NYU, the recruitment is a collaboration between Pulmonary and Thoracic Surgery where they recruit from the bronchoscopy schedule at Tisch and Bellevue, and from the Thoracic Surgery surgical cases. The samples to be collected are right main stem bronchus brushes for genomics (BU-Spira) and proteomics (Vanderbilt-Liebler), nasal brushings for genomics (BU-Spira), and venous blood for plasma and serum for various types of biomarkers: methylation (Johns Hopkins-Sidransky); cytokines (UCLA-Dubinett); autoantibodies to glycans (NYU-Huflejt); microRNA panel (Ohio State-Croce). To date, all of the sites have completed the IRB approval of the EDRN LTP2 protocol and have begun patient enrollment. Twenty-two patients have been recruited so far. The Project will assay all biomarkers for lung cancer that have been reported from airway and blood in a common format, highlighting the need for a resolution of the dilemma of the indeterminate nodules: nodules larger than the above range are most likely cancer, while those below that range are most likely benign and ould be followed. The importance of this project stems from NLST where 25% of the study subjects had a non-calcified nodule discovered on CT-scan of the chest. All subjects on this study will have a CT-scan and the evaluation of the nodules will cover all characteristics using 1.5 mm as a cut-off for Vanderbilt radiologists to develop a radiomics platform to distinguish benign from malignant nodules.

References

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SRM-MS and TMA for Biomarker Measurement Outcome and Association

Leading PI: Alvin Liu, Ph.D., University of Washington

This work involves collaboration among UW BDL, UCLA BRL, Johns Hopkins BRL, PNNL BRL, and UTHSCSA CVC. This project was based on Dr. Alvin Liu's study which

demonstrated differential expression of several markers in prostate cancer cells and cell lines. Those included secreted proteins such as AGR2, KLK3 (PSA), ACPP, MSMB, AZGP1 and a membrane antigen CD10. Interestingly, the expression of membrane protein CD10 was highly correlated with a higher Gleason score and lymph node metastasis. This team project was focused on the development of quantitative assays for detection of aggressive prostate cancers. Monoclonal antibodies specific for AGR2 were developed for IHC detection as well as ELISA. Affinity-free detection of the AGR2 protein was developed using PRISM-SRM-MS; and additional RNA based detection using Nanostring technology. Also urine and blood based assays were developed. Based on the initial observations, the team screened 200 radical prostatectomy specimens using TMAs developed by the UCLA BRL (David Chia). This data indicated that $CD10^{high}$ $AGR2^{low}$ phenotype was most frequent in high grade primary tumors. Conversely, bone and other soft issue metastases and derivative xenografts, expressed more AGR2 and less CD10. AGR2 was readily detected in tumor metastases. However, $CD10^{low}AGR2^{high}$ phenotype is more common in metastases. It appears that AGR2 has a "protective function" in primary tumors but may have a role in the distal spread of the tumor cells. The preliminary data using the SRM-MS quantitative measurements is very promising, but was based on a relatively small sample size. In the next phase, the investigators will expand their analysis using a larger cohort to determine whether these markers add value to the existing gold standard, PSA testing for the detection and diagnosis of prostate cancer. The TMA study was well powered and provided high quality data.

Reference

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Urinary RNA testing to Reduce Over-Detection and Enhance Identification of Aggressive Prostate Cancer; a Prospective, Multi-Center NCI-EDRN Study

Leading PI: Martin Sanda, M.D., Emory University

This study is a good, concrete overview of the EDRN pipeline where significant discoveries start at the EDRN BDLs and progress all the way to large validation trials. . The EDRN BDL (PI: Arul Chinnaiyan) worked with industry to develop a robust TMPRSS2-ERG clinical assay. The EDRN CVCs strategically developed a validation reference set prospectively collected for the PCA3 validation study (involving EDRN CVCs of Martin Sanda, John Wei, Ian Thompson and 10 additional medical centers). Martin Sanda's CVC and Arul Chinnaiyan's BDL conducted the TMPRSS2-ERG prevalidation study and worked with the DMCC to develop the models to build a test that will have clinical value if validated. The industrial partner, Hologic GenProbe, transferred the assay to an EDRN BRL (Daniel Chan) for the validation trial. The study was coordinated by the CVC (Martin Sanda) and DMCC (Ziding Feng) with respect to specimen blinding, QC, model building, and data analysis. The multicenter validation study is at the finish line and samples are being tested by the BRL.

The discovery of TMPRSS2-ERG is one of the most important discoveries in prostate cancer in the past decade. Without partnership with Hologic Gen-Probe this study would not be possible through support by an NIH funding mechanism alone. If validated and put into clinical use it has the potential to spare up to 40% of men from unnecessary biopsies, while maintaining 95% sensitivity for Gleason 7+ prostate cancers.

Reference

Sanda MG. Combining urinary detection of TMPRSS2:ERG and PCA3 with serum PSA to predict diagnosis of prostate cancer. *Urol Oncol*. 2013 Jul;31(5):566-71.

Circulating Biomarkers for Early Detection of Triple Negative Breast Cancer (TNBC)

Leading PI: Karen Anderson, M.D., Ph.D., Arizona State University

The goal of this team project is to discover and validate blood-based markers for the early detection of breast cancers that are negative for the ER, PR and Her2-neu receptors - Triple-negative breast cancer (TNBC). The latter is typically aggressive, not detected by mammography, occurs with higher frequency in younger, pre-menopausal women and is more frequent in black versus white women. The study design focuses on the identification of three distinct types of blood-based biomarkers: autoantibodies (using the NAPPA platform developed by EDRN PI: Josh LaBaer), protein antigens, and miRNA. Multiple biomarkers contributed by investigators in two EDRN BDLs (ASU and FHCRC) and a non-EDRN lab (PNNL) will be validated in a step-wise fashion using samples provided by two CVCs (Duke U., and FCCC), NCI's CPTAC, and multi-institutional cohorts, with study design and data analysis conducted by the DMCC. Aim 1. Verify novel biomarkers for TNBC on a common set of blinded samples; Aim 2. Validate the top biomarkers for TNBC using a diagnostic, PRoBE-compliant set of plasma; Aim 3. Determine the sensitivity, specificity, and PPV of the top marker combinations found in aim 2 to distinguish TNBC from benign breast disease, and for the detection of ER+ and Her2+ breast cancer, using the EDRN Breast Reference Set. Aim 4. Develop a phase 3 validation plan for testing the top biomarkers and biomarker combination(s) for TNBC detection using pre-diagnostic sera from PLCO, ROCA, and/or WHI.

The discovery phase is complete and a phase 2 prevalidation study is almost complete. There are roughly 14 investigators involved with this project. Eighty biomarkers (split into two sets: A and B, based on potential and for priority in data analysis) are being tested on two sets of blinded samples (from DUMC and CPTAC, total=64 cases and 190 benign breast disease controls). The plasma samples being tested have been carefully selected and matched by age and gender to cases, and distributed blinded in 3:1 control:case ratio. All samples are being run simultaneously in the different labs. With respect to the data, so far from ASU, the data are highly consistent across the samples. The investigators do not know the case/control status at this point, but signals are clearly evident in a subset of samples, to several of the A list antigens. Of the participating three assay labs, data collection has been completed and DMCC is conducting the data analysis. In addition, analysis of the breast cancer reference set showed that CA-125 is elevated in TNBC

so the collaborative group has added CA-125 to the list of biomarker candidates. The DMCC has helped set targets for biomarker performance (98% specificity and 38% sensitivity) based on clinical considerations, it has been instrumental in designing the phase 2 study, and has facilitated distribution of blinded samples. Based on the performance metrics of the top performing biomarkers, the investigators will proceed with testing the EDRN reference set to assess performance with other breast cancer subtypes, and they will work with PLCO/ROCA/WHI to identify pre-diagnostic samples and the study design for a validation study. It is anticipated that, if successful, these will become the first validated blood-based biomarkers for stratifying women at increased risk of TNBC who can benefit from more frequent screening by an imaging modality such as mammography or MRI.

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