
The Early Detection Research Network's Specimen Reference Sets: Paving the Way for Rapid Evaluation of Potential Biomarkers

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BACKGROUND: The mission of the National Cancer Institute's Early Detection Research Network (EDRN) is to identify and validate cancer biomarkers for clinical use. Since its inception, EDRN investigators have learned a great deal about the process of validating biomarkers for clinical use. Translational research requires a broad spectrum of research expertise, and coordinating collaborative activities can be challenging. The EDRN has developed a robust triage and validation system that serves the roles of both "facilitator" and "brake."

CONTENT: The system consists of (a) establishing a reference set of specimens collected under PROBE (Prospective Specimen Collection Retrospective Blinded Evaluation) design criteria; (b) using the reference set to prevalidate candidate biomarkers before committing to full-scale validation; (c) performing full-scale validation for those markers that pass prevalidation testing; and (d) ensuring that the reference set is sufficiently large in numbers and volumes of sample that it can also be used to study future candidate biomarkers. This system provides rigorous and efficient evaluation of candidate biomarkers and biomarker panels. Reference sets should also be constructed to enable high-quality biomarker-discovery research.

SUMMARY: We describe the process of establishing our system in the hope that it will serve as an example of how to validate biomarkers for clinical application. We also hope that this description of the biospecimen reference sets available from the EDRN will encourage the biomarker research community—from academia or

industry—to use this resource to advance biomarkers into clinical use.

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The Early Detection Research Network (EDRN)⁴ is a research consortium established in 1999 by the National Cancer Institute with the mission of translating promising cancer biomarkers to clinics for use in assisting with medical decisions. Of particular interest are biomarkers for cancer risk prediction, diagnosis, early detection, and prognosis. The main driver for establishing the consortium was the observation that patients with early-stage cancer had better survival rates than those with late-stage disease. The assumption was that shifting cancer diagnosis to earlier stages might improve the survival of patients with cancer. In addition, it appeared that many candidate biomarkers had been reported but that clinical usefulness had been validated for very few markers. Given the tremendous advances that had occurred in genomics and proteomics, the hope was that these newly discovered biomarkers would lead to substantial improvements in the early diagnosis and prediction of cancer risk, thereby reducing the burden of cancer on the US population. The mission of the EDRN is to facilitate this process.

Since its inception, EDRN investigators have learned a great deal about the process of validating biomarkers for clinical use. Translational research requires a broad spectrum of research expertise, and coordinating collaborative activities can be challenging. The players include molecular biologists, clinicians, and population scientists, whose cultures and research methods are quite different. Nevertheless, with a goal in common, we have established a robust system for

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⁴ Nonstandard abbreviations: EDRN, Early Detection Research Network; MS, mass spectrometry; PSA, prostate-specific antigen; hk2, human kallikrein 2; DMCC, Data Management and Coordinating Center; PROBE, Prospective Specimen Collection Retrospective Blinded Evaluation.

biomarker triage and validation that facilitates choosing and rigorously validating good biomarkers. We describe the process of establishing our system so that it can serve as an example of how to validate biomarkers for clinical application. We also describe the biospecimen reference sets that are available in the EDRN to encourage the biomarker research community—from academia or industry—to use this resource to advance their biomarkers into clinical use.

SELDI-TOF Mass Spectrometry Validation Study Experience

During the years 2000 to 2002, many reports appeared in the literature on protein profiling with SELDI-TOF mass spectrometry (MS) for cancer diagnosis. Because many of these reports strongly indicated a potential for clinical utility (1–3), the EDRN decided to validate SELDI-TOF-MS protein profiling for prostate cancer diagnosis. Because this technology was unconventional, in that it did not identify informative proteins but relied on a protein MS pattern to distinguish cancer patients from controls, EDRN investigators opted for a 3-stage validation process (4). The first stage was to demonstrate that SELDI-TOF-MS protein profiles applied to the same serum pool are reproducible across different laboratories. After many efforts at standardization, this stage was successfully completed (5), demonstrating that the profiles were indeed reproducible. The second stage was to use high-quality samples from a well-designed multicenter study to determine whether the serum profile could distinguish individuals with biopsies positive for prostate cancer from individuals with negative biopsy results. The results were negative (6); the study did not reproduce previously identified informative peaks or identify new informative peaks that distinguished prostate cancer patients from patients with negative biopsy results. The third stage, to determine whether prostate cancer could be detected early, would have been based on sera from the Prostate Cancer Prevention Trial, but that study was not pursued.

The first 2 stages of this SELDI-TOF-MS validation study taught investigators several important lessons. The first lesson was the identification of an important source of bias that may have been responsible for early “promising” results that subsequently did not lead to validation. This biasing factor was the differences in the lengths of time and conditions under which serum samples were stored for prostate cancer cases and nonprostate cancer controls (7). It turned out that protein peak intensities were negatively associated with storage length (7). Unfortunately, serum samples from cancer patients tended to be collected over many years and used multiple times, whereas sera for controls tended to have been collected recently and

subjected to fewer freeze–thaw procedures. In the stage 2 validation study that EDRN conducted, case and control samples were chosen to be similar with regard to storage time and the number of freeze–thaw cycles (no more than a single freeze–thaw), as well as to be similar to other clinical and epidemiologic factors (age and race).

The second lesson was the crucial importance of having available an unbiased well-designed set of samples to evaluate a marker. Although we suspected the source of bias in the promising preliminary studies, we needed high-quality samples to definitively test the marker. Two years were required to identify the number of individuals with samples in existing repositories who would satisfy the tight inclusion and exclusion criteria. We could have avoided this 2-year delay in evaluating the value of the SELDI-TOF-MS marker had a reference set been available.

Establishing a Prostate Cancer Reference Set and the Reference Sets for Other Organ Groups

In 2004, the EDRN’s Genitourinary Collaborative Group decided, after lengthy discussions, to establish a prostate cancer reference set that could address the various issues identified in the SELDI-TOF-MS validation study. The first decision was that the reference set should be designed with a clear clinical application in mind and that samples should be collected from the target clinical population without any potential for bias. Three clinical application settings were identified. The first setting was to help men who were candidates for biopsy under the current clinical guidelines decide whether to undergo the biopsy procedure. The intent was to reduce the number of unnecessary biopsies without reducing the detection rate of prostate cancer. The second setting was to help men who had a negative biopsy result decide whether to undergo subsequent biopsies. The third setting was to aid men in making treatment decisions after a biopsy result positive for prostate cancer. Although the third clinical question is probably the most important for the clinical care of prostate cancer, answering this question requires long-term follow-up to collect mortality data. The Genitourinary Group decided to initially focus on the first application, men recommended for their first biopsy. Prostate-specific antigen (PSA) would not be a good marker for this population because these men most likely were candidates for biopsy because of their increased serum PSA concentrations. This comprehensive clinical application presents a realistic and practical problem with potential impact for individuals and for the population.

Moreover, the study design for this application is straightforward. Serum samples could be obtained prospectively from men before biopsy, and outcomes

from the pathology report would be available shortly thereafter. This feature eliminates common sources of biases in case control designs, in which samples are collected after disease status is known.

The second decision was that the reference set would be used for triaging biomarkers. Investigators, within or outside of EDRN, would be invited to submit their biomarkers for a blinded evaluation of the reference set as a prevalidation step. Again, note that if such a reference set had been available, the full-scale SELDI-TOF-MS validation study could have been avoided. A marker with a good performance in the reference set would then undergo a full-scale validation study.

The third decision was that although the sample size of the prevalidation reference set was chosen to be 120 (60 men with a positive biopsy result and 60 men with a negative result), recruitment would continue to allow timely completion of a validation study if some markers were found to merit validation. The unbiased selection of cases and controls, the uniform collection of serum samples, and the fact that the same type of sample was used for prevalidation and validation studies ensure a high probability that the performance of the marker observed in the prevalidation set will hold up in the full-scale validation study and that the biomarker, if validated, will have clinical utility.

In 2005, similar discussions occurred for the other 3 EDRN organ-specific collaborative groups (lung, gastrointestinal, and breast/gynecologic), and a number of reference set studies were designed and executed thereafter. One important development is the growing expectation that any EDRN validation study must also produce a reference set that can be used to validate future biomarkers for the same clinical application. Indeed, this criterion has become important in evaluating and approving proposals for validation studies.

Description of the Existing EDRN Reference Sets

To date, the EDRN has constructed 12 biospecimen reference sets. These sets are summarized in Table 1. Further details can be found on the EDRN website (8).

Use of the Reference Sets to Triage Biomarkers for Validation

The prostate cancer prevalidation set was quickly established with samples contributed from 3 EDRN Clinical and Validation Centers. Biomarker-discovery laboratories were invited to present their markers to the EDRN collaborative group, which ranked them and voted on them for access to the prevalidation set. Markers from 4 laboratories were approved for prevalidation, and aliquots of serum were sent to each laboratory in a blinded fashion. The markers approved for

prevalidation were human kallikrein 2 (hk2), hk4, hk11, thrombospondin 1, percent [−2]proPSA, and EPCA2 (early prostate-specific antigen 2). Assay results were sent to the EDRN Data Management and Coordinating Center (DMCC) for analysis and comparison. Only percent [−2]proPSA passed the prevalidation stage (9), and it went on to a successful full-scale validation study (10). This biomarker received US Food and Drug Administration clearance in 2012.

Widespread PSA screening complicates biomarker evaluation for prostate cancer risk prediction or diagnosis, because a biopsy is usually triggered in current clinical practice by increased serum PSA concentrations. The 3 clinical applications on which the Genitourinary Group has focused are important, given the reality of PSA screening. If the PSA screening patterns or criteria for post-PSA workup change, the target population for application of a biomarker might also change, and the validation results from the current reference set might not be generalizable. On the other hand, constructing a reference set for general population screening would require obtaining biopsies from men who would not undergo biopsy under current practice. This requirement would be feasible only in some large prostate cancer prevention trials that require biopsies from all participants regardless of their PSA values, such as occurred in the Prostate Cancer Prevention Trial. Teaming up with such large cohorts would allow the EDRN to address that question.

Another difficulty in designing a reference set for general population screening of prostate cancer is the high prevalence of indolent prostate cancers. It seems more efficient to first address the third clinical application proposed above and learn more about aggressive prostate cancer before designing a biomarker study for general population screening. Focusing on the clinical applications for which studies are more feasible and more likely to give fruitful results is an important lesson learned in the EDRN.

Design of Reference Sets and Full-Scale Validation Studies—PROBE Design

Most EDRN reference sets are prospectively designed and coordinated with multicenter specimen collections. A protocol and manual of operations were developed for each prospectively established reference set and were reviewed both internally and externally. Training on the manual of operations and site audits for quality control were conducted by the EDRN DMCC staff. The EDRN developed a standard operating procedure that requires blood tubes to sit after collection for 30 to 60 min at room temperature. Then, if the tubes were not processed immediately, they were permitted to be stored at 4 °C for no more than 4 h.

Table 1. Descriptions of the EDRN reference sets.

Reference set name	Clinical context	Study design	Study population	Case/controls	Specimen collected	Sample size	No. of collection sites/ No. of laboratory- tested biomarkers
Lung reference set A	Diagnosis of lung cancer	Case control; matched for age, sex, smoking status, pack-year	All suspicious lung lesions on CXR ^a or CT	Lung cancer $\geq 50\%$ stage I/free of lung cancer at 1 year after blood draw	Serum, plasma	207 Cases, 230 controls, 137 other cancer controls	8/5
Lung reference set B	Early detection of lung cancer	Case control; matched for age, sex, smoking status, pack-year	CT screening	CT-detected lung cancer >0.5 cm and <3 cm/ lung nodule >0.5 cm and <3 cm, free of lung cancer at 1-year F/U CT	Serum	38 Cases, 87 controls, 25 other cancer controls	4/0
Lung nodule study ^b	Interpreting CT-identified lung nodules	Prospective	CT screening	Smokers with indeterminate pulmonary nodules (5–20 mm) by CT	Serum, plasma, endobronchial brushing, nasal brushing	Targeted cohort of 200; expect 30 lung cancer cases at 2-year F/U; 170 controls	4/0
Percent [–2] proPSA prostate cancer validation study	Diagnosis of prostate cancer	Prospective	Consecutive men scheduled for prostate biopsy	Biopsy results positive for prostate cancer/biopsy results negative for prostate cancer	Serum	245 Cases, 321 controls	4/1
PCA3 prostate cancer validation study	Diagnosis of prostate cancer	Prospective	Consecutive men scheduled for prostate biopsy	Biopsy results positive for prostate cancer/biopsy results negative for prostate cancer	Urine, serum, plasma	332 Cases biopsy positive for prostate cancer, 538 cases biopsy negative	10/2
MSA bladder cancer validation study	Detection of bladder cancer	Prospective	Patients with incident or recurrent superficial bladder cancer	Recurrent bladder cancer at 2-year F/U/without recurrent bladder cancer at 2-year F/U	Urine, plasma, serum	81 Recurrent bladder cancers, 189 no-recurrence controls; 100 additional baseline normal controls, 100 controls with benign conditions	18/1
GLNE10 vimentin methylation in colon cancer validation study ^b	Detection of colorectal cancer	Prospective	Patients scheduled for colonoscopy without clinical indication	Colorectal cancer or HGD /adenoma >1 cm/ neither	Stool, plasma, serum, urine	6000 Targeted colonoscopies; expect 90 colorectal cancers or HGD, 450 adenomas >1 cm; 5460 controls	30/0
Pancreatic cancer reference set	Diagnosis of pancreatic cancer	Case control; matched by age, sex, race, study site	Newly diagnosed pancreatic cancer patients matched with healthy and benign controls	Patients with stage I or IIA pancreatic cancer/ chronic pancreatitis/ acute benign biliary obstruction/healthy controls	Serum, plasma	57 Pancreatic cancers, 63 chronic pancreatitis cases, 31 cases of acute biliary obstruction, 61 healthy controls	5/1

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Table 1. Descriptions of the EDNRN reference sets. (Continued from page 71)

Reference set name	Clinical context	Study design	Study population	Case/controls	Specimen collected	Sample size	No. of collection sites/ No. of laboratory- tested biomarkers
Pancreatic cystic fluid reference set ^b	Predict malignant potential of pancreatic cystic lesion	Prospective	Patients with pancreatic cysts and scheduled for workup (EUS or surgery)	Malignant or high potential for malignant cysts/low potential for malignant cysts/benign cysts with no malignant potential	Serum, plasma, cystic fluid	Targeted cohort of 450; expect 185 cases, 130 of low malignant potential, 135 benign	7/0
Liver cancer validation study (DCP)	Diagnosis of early HCC	Case control; matched for age, sex, viral etiology	Cirrhotic patients	Consecutive newly diagnosed HCC/cirrhotic patients	Serum, plasma, DNA	419 HCC cases (211 later stage, 208 early stage), 417 cirrhosis controls	7/7
Liver cancer validation study (HEDS) ^b	Early detection of HCC	Prospective cohort study	Cirrhotic patients	HCC incidence during F/U/free of HCC during F/U	Serum, plasma, DNA	Targeted cohort of 1110 cirrhotic patients; expect HCC incidence of 85	7/0
Breast cancer reference set	Recommend women with breast lesion for biopsy or not	Prospective	Patients scheduled for breast biopsy diagnosis	Biopsy-confirmed invasive breast cancer/benign breast disease	Serum, plasma	207 Invasive breast cancer cases, 294 cases of benign disease, additional 55 cases of in situ cancer, 276 controls	4/2

^a CXR, chest x-ray; CT, computed tomography; F/U, follow-up; PCA3, prostate cancer antigen 3; MSA, microsatellite analysis; GLNE10, Great Lakes New England Clinical Validation Center Protocol #10; HGD, high-grade dysplasia; EUS, endoscopic ultrasound; DCP, Validation of Serum Markers for the Early Detection of Hepatocellular Carcinoma; HCC, hepatocellular carcinoma; HEDS, Hepatocellular Carcinoma Early Detection Strategy study.
^b Study is ongoing.

Long-term storage is at a temperature of -80°C or colder. The sample-processing requirements vary for some reference set protocols, but the information and the standard operating procedure are all available on the EDRN public portal (8). The adherence to the protocols is usually excellent for prospective studies coordinated by the DMCC because of the training and auditing. Actions have been taken when deviations have been identified. In one study, the DMCC suspended a collection site and did not use the samples because its audit discovered serious deviations from protocol. The extent of adherence to the protocol varies for retrospectively constructed reference sets, i.e., sites identifying existing samples that satisfy the protocol. The DMCC has been examining all retrospectively constructed reference sets and documenting identified protocol deviations. The findings will be added to the EDRN public portal describing that reference set. Samples were shipped to the Frederick National Laboratory of the National Cancer Institute, and the EDRN's Validation Study Information Management System was used to store clinical data centrally at the DMCC. Most EDRN reference sets adhere to the design criteria of the PRoBE (Prospective Specimen Collection Retrospective Blinded Evaluation) study (11) and are therefore strongly unbiased for their intended clinical application context (i.e., samples collected from a clinically relevant and well-defined cohort in the absence of knowledge about patient outcome). Constructing PRoBE-designed reference sets is efficient because the lengthy sample-collection period can begin even before markers become available for testing. For example, the industrial partner who owns the license for the percent $[-2]$ proPSA marker included a subset of the data from the EDRN reference set in their Food and Drug Administration clearance trial (they restricted the range of PSA and thus did not include the entire reference set). Moreover, combinations of markers tested at different times but on the same reference set specimens can be evaluated with such reference sets. Another attractive feature of PRoBE-designed reference sets is that they avoid biases and ethical issues associated with studies that obtain biomarker test results at the time of recruitment, in which diagnostic workup and patient management may be influenced by biomarker values. In the PRoBE design, biomarker tests are obtained retrospectively after the patient has been treated. Details and an extensive discussion of the PRoBE design are available (11).

Future Directions

Discussions on expanding the use of reference sets for biomarker-discovery research are currently under way. One striking observation from our experience with ref-

erence sets is the drastic decrease in performance of many biomarkers that show very promising results in the discovery phase. As we have observed, bias from samples used in discovery studies is difficult to disentangle if the collection of samples does not meet PRoBE design criteria. Although it is helpful that the EDRN has reference sets to prevalidate these markers and eliminate false leads, it would be more efficient if biomarkers that moved out of the discovery phase had a higher chance of retaining their performance. The use of high-quality samples that come from the population targeted for the intended clinical application at the discovery phase will increase the chances of better biomarkers moving into the validation pipeline, thereby increasing the chances of successful validation.

Even good reference sets have limitations. For example, they are derived from specific institutions, and the performance results observed for the biomarker may not be generalizable to other populations. Consequently, external validation of markers is also necessary. For the EDRN prostate cancer reference set, the prevalence of a positive biopsy result is $>40\%$, somewhat higher than in the general population of men who currently undergo biopsy for prostate cancer diagnosis at their local clinics. This finding suggests that the EDRN reference set may not represent the general population and argues for external validation of biomarkers that validate on this set.

For less prevalent cancers, such as ovarian cancer and pancreatic cancer, construction of PRoBE-designed reference sets for early detection may not be feasible at the discovery stage or even at a nonpivotal validation-trial stage. Precious samples are likely to be saved for validation studies. Community healthcare systems have much better long-term follow-up, and results are more generalizable than tertiary healthcare centers. Partnering with them will greatly enhance our ability to establish reference sets for early detection of less prevalent cancers. Even when full adherence to PRoBE standards cannot be achieved, as has occurred with several EDRN reference sets constructed in earlier years, some of the PRoBE design principles can and should still be incorporated into the construction of specimen reference sets. These principles include ensuring that samples are collected according to a rigorous protocol, ensuring that data are documented with respect to factors that might influence biomarker values or disease characteristics, and incorporating samples from multiple centers. Such sample sets would be of much greater value than those that have been used in most discovery studies to date. Blinding and randomization should also be implemented reliably at a central location. This criterion differs from descriptions in many biomarker-discovery reports, in which one can-

not tell whether or how blinding and randomization were carried out.

There should be a policy for access to these high-quality samples for biomarker discovery. The policy could be similar to that used for access to prevalidation sets, except that for the discovery purpose one does not need the preliminary data indicating a potential clinical application. The policy should have elements, however, that include a strong rationale for the approach, a strong design for the proposed study, evidence of a robust assay, and agreement that samples will be blinded before the assay is performed, with unblinding occurring only after the assay results have been sent to the central data repository and archived for later combination with other markers for constructing marker panels. Panel construction can be postponed for a certain period to allow the marker developers to exploit the intellectual capital invested in their markers, but a plan should be in place to use the reference set data to try to identify panels with significantly better performance than any single marker. The availability of high-quality samples for biomarker discovery, triage, and prevalidation, and validation in a coordinated system will en-

hance the translation of biomarkers from bench for clinical use and the benefit of patients.

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