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Moonshot Objectives: Catalyze New Scientific Breakthroughs – Proteogenomics

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Abstract

Breaking down the silos between disciplines to accelerate the pace of cancer research is a key paradigm for the Cancer Moonshot. Molecular analyses of cancer biology have tended to segregate between a focus on nucleic acids – DNA, RNA and their modifications – and a focus on proteins and protein function. Proteogenomics represents a fusion of those two approaches, leveraging the strengths of each to provide a more integrated vision of the flow of information from DNA to RNA to protein and eventually function at the molecular level. Proteogenomic studies have been incorporated into multiple activities associated with the cancer moonshot, demonstrating substantial added value. Innovative study designs integrating genomic, transcriptomic and proteomic data, particularly those using clinically relevant samples and involving clinical trials, are poised to provide new insights regarding cancer risk, progression, and response to therapy.

Keywords

proteogenomics; proteomics; cancer moonshot; mass spectrometry

The stated objective of the Cancer Moonshot is enormous: ‘to end cancer as we know it’ by doubling the rate of scientific progress and its translation into patient care in the near term. The core hypothesis is that these goals cannot be achieved by existing technologies as currently applied, but rather will require a focused investment in specific technologies that can provide insights greatly advancing our ability to understand, predict, and control the malignant progression of cancer.

Chief among the scientific breakthrough that have set the stage for the Cancer Moonshot, is the revolution in our molecular understanding of cancer that began with the sequencing of the human genome in 2001[1] and continued through the Cancer Genome Atlas (TCGA) [2,3], the ENCODE project [4], the 1000 Genomes Project [5] and the International Cancer Genome

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Consortium [6]. While TCGA has delivered complex genomic datasets, including DNA, mRNA, DNA methylation, and limited protein analysis on over 10,000 tumors from 32 different tumor types [7], there is still much to learn about the complex biology linking mutations, copy number aberrations, and epigenetic modifications to RNA transcription and to changes in protein abundances, activities, and ultimately function.

Proteogenomics is an emerging discipline focused on the integration of comprehensive genomic and proteomic data from the same source – tumor or patient [8–11]. Mass spectrometry-based proteomics is based on the amino acid sequence of peptides and proteins [12], and thus totally dependent on the detailed knowledge of the human genome and its modifications in cancer as provided by the Human Genome Project and by TCGA. Predicted amino acid sequences derived from whole exome sequencing (WES) or whole genome sequencing of tumor DNA can be searched against the mass spectra data, providing verification for the expression of predicted aberrant proteins [13,14]. In addition. the effects of post-transcriptional regulation on protein abundance can be monitored through parallel measurements of mRNA and protein abundance. Proteomics can also provide direct measurements of post-translational modifications with a direct bearing on protein function, including not only phosphorylation, but also glycosylation, acetylation, O-GlycNAcylation, ubiquitinylation, methylation, and sumoylation.

The distinguishing feature of proteogenomics is the integration of all these components into a more systems-level view of tumor biology, as illustrated by the initial three publications resulting from the National Cancer Institute's Clinical Proteomic Tumor Analysis Consortium (CPTAC) analysis of colorectal, breast, and ovarian tumors previously analyzed by TCGA [15–17]. Initial comparisons of mRNA and protein abundance in the same specimens revealed the complexity of the relationship between mRNA and protein abundance. While the mean Spearman's correlation coefficient was 0.23 for mRNA-protein pairs across colorectal samples [16], and similar correlation coefficients of 0.39 and 0.45 were observed for breast and ovarian tumors [15,17], respectively, all three studies observed a range of correlation coefficients, from slightly negative to approximately 0.85. Interestingly, all three studies showed similar patterns of functional association, in which housekeeping proteins (ribosomes, oxidative phosphorylation, mRNA splicing) had the lowest correlation coefficients, while proteins associated with short term responses (inflammatory response, amino acid and nucleotide metabolism) showed a high degree of correlation [15–17]. Similar observations have been made in healthy tissues[18,19] implying that this relationship reflects a functionally important level of regulation.

Proteogenomics can also confirm the expression of protein variants predicted by genomic measurements, classified by the type of genomic variant. In the breast cancer cohort, 4.1% of nonsynonymous, single nucleotide variants were observed in the proteome data, but only 0.28% of novel RNA splice junctions, not represented in RefSeq [15]. Since these analyses require the addition of the predicted variants to the databases against which mass spectra are searched, false discovery can be a serious problem in a dataset with millions of spectra and an enlarged sequence search space. This concern stimulated the development of new tools and understanding to control false discovery rates [13,20]. Even so, interpreting the failure to detect predicted genomic variants is a complicated issue: does the failure to detect mean the

variant is not expressed, or present at a low abundance, beneath even the improved sensitivity of current MS platforms? And if particular classes of variants are truly under-represented at the protein level, are there functional consequences? Orthogonal measurements, including targeted proteomic assays and antibody-based approaches, will be required to answer these questions definitively.

Perhaps the most impactful contribution of mass spectrometry-based proteomics is the ability to provide a comprehensive analysis of protein phosphorylation, including phosphorylations on serine, threonine and tyrosine, without the need to restrict analyses to only those targets with qualified antibodies, which is the major limitation of the reverse phase protein array (RPPA) methodology used in TCGA. In the ovarian cancer study, 2,324 differently phosphorylated proteins were identified in short versus long survivors, in comparison with the 31 phosphoproteins that could be interrogated using RPPA on the same samples [17]. Although the protein and phosphoprotein targets accessible via RPPA represent some of the most significant cancer-associated pathways in the curated literature [21–23], the ability of mass spectrometry to identify thousands of phosphosites enabled integration across multiple proteins and phosphoproteins to identify increases in the activity of pathways considered as a whole, even if individual components had only moderate p values. This is well illustrated in the ovarian cancer report, where activation of the platelet-derived growth factor receptor (PDGFRB) pathway, assessed by integration of protein abundance and phosphorylation across multiple components, was found to be significantly associated with short overall survival, even though relatively few individual events in the PDGFRB pathway were statistically significantly different between the short and long surviving groups [17]. These results highlight that the overall functional activity of a pathway results from integrating both protein and phosphoprotein abundance, and that consistent trends in the larger set of proteins that comprise a pathway are informative when integrated across all components of the pathway. This is not a surprising finding in cancer where pathways as a whole are commonly dysregulated.

Challenges and Opportunities in Proteogenomics

Sample size

One major distinction between genomic and proteomic measurements lies in sample size requirements. The availability of nucleic acid amplification techniques, including polymerase chain reaction (PCR) and its multiple variants, has enabled accurate measurements of increasingly small samples, to the point that single cell DNA and RNA measurements are now routinely achievable [24–26]. The lack of a similar method for direct amplification of proteins presents a major challenge for proteomic measurements, particularly for quantification of low abundance proteins and low frequency post-translational modifications. Although continuing technical improvements in the sensitivity of mass spectrometers have produced a steady decrease in the amount of sample required, sample size remains a limiting factor that complicates analysis of clinically relevant samples such as needle biopsies and laser microdissected tissue samples. While targeted MS approaches such as immuno-MRM [27] and PRISM-SRM [28] have increased sensitivity and thus decreased sample size requirements, the selection of targets requires *a priori* knowledge

based on discovery experiments using either transcriptomic methods or global mass spectrometry approaches on larger samples. Thus many mass spectrometry laboratories are actively engaged in technology development focused on the analysis of ultra-small samples of 100 or fewer cells [29–35]. While true single cell sensitivity is not required for clinical samples such as needle biopsies, the ability to provide deep proteomic and phosphoproteomic coverage from fewer than 100 cells will open up the direct clinical application of proteogenomics.

Increased proteomics measurements throughput

Another major distinction is that the throughput of the proteomics measurements compared to genomics is far smaller, and presently along with sample size requirements greatly limits the speed and scope of application. Proteomics achieves broad coverage of proteins and protein modified proteins by the use of sample fractionation; in essence, each sample is made into many smaller samples. The result of this is to not only increase study costs, but also to decrease the effective throughput as well as the minimum sample size requirements. The constraints for present mass spectrometry based proteomics arise due to a combination of factors, including the speed and resolution of the separation methods used in conjunction with (almost exclusively liquid chromatography) and the sensitivity and dynamic range of the mass spectrometry technology. Major gains in these capabilities are being pursued, and are anticipated due to developments that greatly speed the separations used, such as those based upon gas phase ion mobility separations [36], and recent advances that are also increasing the resolution of the separations, as well as the sensitivity of the measurements and doing this in conjunction with much faster forms of mass spectrometry [37].

Tumor heterogeneity

As the spatial resolution of genomic measurements has increased, it has become increasingly evident that tumors are highly heterogeneous, both in the composition of distinct cell types into a tissue, and in the mutational complexity and clonal structure of the malignant epithelial cells comprising the tumor [26,38–42]. Laser microdissection (LMD), in the hands of an experienced pathologist, permits the interrogation of anatomically distinct portions of a tumor or tissue as distinct entities, allowing separate molecular analyses of tumor epithelial and stromal components, and furthering our understanding of tumor – microenvironment interactions[43,44]. LMD can also be used for spatially distributed sampling within histologically similar areas of a tumor, thus providing insight into the degree of molecular heterogeneity within tumors, even in seemingly homogeneous [40,45].

The ability to amplify the nucleic acid target using PCR and its derivatives, combined with increasingly sophisticated methods for laser microdissection of tumors, have provided spatially resolved genomic and transcriptomic analysis of tumors that distinguishes clearly between the epithelial and stromal compartments of a tumor, and differentiates among distinct cell types within the stroma [44]. Single cell analysis of dispersed epithelial cells has provided significant insight regarding the clonality and genetic diversity of tumors [41,42]. Attempts to increase the spatial resolution of proteomic measurements inevitably confront the trade-off between available sample and depth of coverage. The deep proteomic coverage (exceeding 10,000 proteins per tumor) attained in the three flagship CPTAC studies required

~20 ug protein as starting material, while a typical 200–300 micron diameter, 10 micron thick LMD section provides only 0.1 ug, yielding at best 1500 to 2000 confident protein identifications. Phosphoproteomic measurements require 10 times as much starting material for the same depth of coverage, due to the low frequency of phosphopeptides as a percent of total peptides. Consequently, high spatial resolution phosphoproteomic studies will require a transformational advance in the ability to analyze small samples, but will enable the inclusion of global phosphosite data in evolving atlases of human cancers.

Archived retrospective samples

The ability to develop targeted therapies for cancer depends heavily on the ability to identify functional changes that not only distinguish between the normal and malignant tissues, but that also reflect clinical outcomes. However, for most cancers, the time period between initial diagnosis and significant clinical outcomes, including disease recurrence or death, is sufficiently long that prospective clinical trials involving fit-for-purpose tissue samples are extraordinarily expensive. In this regard, the availability of biorepositories containing archived formalin fixed, paraffin embedded (FFPE) tissue blocks, obtained at the time of initial diagnosis (i.e., from residual tissue remaining after pathology review for diagnosis) and archived with appropriate metadata and clinical follow-up, provide an invaluable resource for correlative studies on a population scale, eventually leading to the development of prognostic and theranostic biomarkers[46].

The main challenge for proteomic analysis of FFPE specimens is the inherent and significant cross-linking between proteins and other molecules in the tissue through Schiff base formation. This cross-linking hinders efficient and reproducible extraction of proteins, and the presence of chemical modifications impedes peptide identification [47]. Thus, effective reversal of protein modifications is vital to successful quantitative analyses. Due to the considerable interest in archived FFPE samples, significant effort has been directed at addressing these challenges. A number of protein extraction strategies have previously been reported in the literature [27,48,49]. When applied to extensively annotated FFPE archives such as the SEER biorepository [50], deep proteogenomic analysis of FFPE samples can provide robust linkages to clinical outcomes in significantly sized cohorts.

Proteogenomics and the Cancer Moonshot

One of the most significant contributions of NextGen sequencing technologies has been the ability to provide comprehensive whole exome and transcriptome information on patient samples, in a cost- and time-effective manner such that the information is clinically actionable. The emerging field of Precision Oncology is heavily dependent on the identification of driver mutations and transcriptional signatures that are then used to determine the next course of treatment, building on the initial success of imatinib as a specific therapy for BCR-ABL positive chronic myelogenous leukemia patients in 2001 [51]. This basic approach has been extended to an ever-growing list of mutations and selective inhibitors as exemplified by ‘smart’ clinical trials [52–56]. However, results from this approach have been mixed; response rates have hovered around 30% in clinical trials of targeted therapies conducted between 2007–2010, and half of the approved drugs had an overall

response rate of 20% or less [5758]. Clearly the precision medicine community does not yet have a sufficiently detailed picture of the complex biochemical interactions driving drug response and the development of drug resistance, and no single technology is likely to provide the needed detail. Integration of proteomic and genomic data is key to elucidating the complex biology determining tumor responses to selective inhibitors and the multiple compensatory mechanisms leading to drug resistance. Integrating comprehensive proteogenomic data will help design rational approaches to interpret the response of patients to therapeutic agents administered as part of clinical trials, and to build predictive models of response and resistance. Application of proteogenomic methods in mechanistic experiments using preclinical model systems such as organoids or patient-derived xenografts has the potential to build predictive models of the cellular response to selective inhibitors, alone and in combination, enabling a rational approach to combining therapeutic agents so as to minimize drug resistance.

One of the major goals of the Cancer Moonshot is to break down the silos between scientific disciplines in order to accelerate the pace of cancer research. Clearly, proteogenomics provides a bridge between the disciplines of genomics and more traditional biochemistry and enzymology. This potential to catalyze cancer discovery is already recognized by several Cancer Moonshot-associated projects. The CPTAC program has expanded to include a set of Proteogenomic Translational Research Centers focused on the problem of predicting drug response and mitigating drug resistance by working closely with on-going NCI-sponsored clinical trials involving targeted therapeutics. The three currently funded studies are tackling breast cancer, ovarian cancer, and acute myelogenous leukemia by conducting proteogenomic analyses before and after drug treatment, either in the context of neoadjuvant treatment or longitudinal sampling. Pre-clinical models including patient-derived xenografts and *ex vivo* drug sensitivity assays are being used in parallel to test the hypotheses generated from clinical observations and develop molecular signatures that can predict response [https://proteomics.cancer.gov/programs/cptac/consortium/cptac-teams].

In a unique research partnership representing the breakdown of organizational silos, the NCI, the Department of Defense (DoD) and the Veterans Administration (VA) are partnering in the Applied Proteogenomics OrganizationaL Learning and Outcomes (APOLLO) project [9], in which over 8000 annotated human tissue specimens form the VA and DoD medical systems will be subjected to proteogenomic profiling, in a workflow that can be adapted to DoD and VA clinics to match tumor types identified by genomic and proteomic profiling with the appropriate targeted therapies. Initial studies are focusing on lung, ovary, endometrium, prostate, and breast cancers.

A separate collaboration between the DoD's Murtha Cancer Center, the NCI, and a DOE national laboratory, Pacific Northwest National Laboratory, is leveraging the DoD's unique longitudinal collection of serum and tissue samples from military personnel to conduct a longitudinal study of changes in blood proteins preceding the development of clinically evident cancer, at diagnosis, and two years after treatment. The study capitalizes on the Framingham-like nature of the DOD Serum Repository, in that a very large cohort of healthy individuals have been gathered prospectively in a single health care system with common protocols and electronic health records[59,60]. Subjects remain within this health care system

for as long as they are on active military service, and many remain in the system at retirement. By monitoring serum proteins at 4 years and 2 years prior to diagnosis and at diagnosis, this study is poised to capitalize on emerging data indicating the power of 'biomarker velocity', or the rate of change in a serum protein, as sensitive indicators of risk and early diagnosis [61–65]. The availability of consistent follow-up after cancer diagnosis and matched serum samples before and after treatment has the potential to provide biomarkers of response to therapy, and emerging drug resistance. The initial Framingham-like study will focus on head and neck squamous cell carcinoma (HNSCC), including a comparison of human papilloma virus positive and negative subjects, with and without HNSCC. Subsequent studies are planned to investigate diffuse large B cell lymphoma, bladder cancer, and melanoma.

Proteogenomics has become an international endeavor, with the formation of the International Cancer Proteogenome Consortium, which establishes a formal relationship between the National Cancer Institute and 15 institutions in eight countries, including Canada, China, Germany, South Korea, Taiwan, and Switzerland [66]. The consortium is united by the use of integrated proteomic and genomic approaches to study those cancers particularly relevant to each respective country, while adopting common standards for quality control and data sharing.

Conclusion

The emerging field of proteogenomics has clearly demonstrated the added value of combining comprehensive quantitative protein measurements with whole exome sequencing and whole RNA sequencing to provide a truly integrated picture of the complex downstream effects of oncogenic mutations. In doing so, proteogenomics has helped to identify new strategies for early diagnosis, prognosis, and selection of targeted therapies. This marriage of genomics and proteomics is a stellar example of 'breaking down silos', and the synergistic gains available when distinct disciplines coordinate their efforts. In the context of 'catalyzing scientific breakthroughs', mass spectrometry-based proteomics is currently subject to several technical limitations that restrict the clinical utility of proteomics. Chief among these are sample size requirements, which in turn affect the feasibility of analyzing laser microdissected samples from FFPE samples. Solving these technical challenges has the potential to move proteogenomics from a research tool to a routine clinical measurement, and an integral component of precision medicine and smart clinical trials.

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