

Gene Expression Signatures – *Ex Vivo* / *In Vitro* Approaches for Signature Development and Validation

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ABSTRACT Tumor heterogeneity has warranted the development and validation of gene expression signatures. This approach will help the oncologist in improving diagnosis and/or prognosis. Chronic Inflammation is considered to be involved in neoplasia and commonalities have been observed, in the wound healing response as well as in tumorigenesis. In this regard, the serum response of fibroblasts was shown to mimic the *in vivo* wound healing response in terms of the “wound response signature” using a variety of molecular biological approaches. Correlation of such signatures, using samples obtained by *ex vivo* and *in vitro* methods, developed and/or validated by a variety of genomic, transcriptomic and proteomic approaches with the clinical outcome, will enable the researcher and the oncologist/clinician to interface and potentially develop and validate cost-effective methods of diagnosis potentially leading up to personalized therapy. Patient selection and stratification and sample size considerations are paramount in developing and validation of improved genomic classifiers.

INTRODUCTION

Anecdotal information, case reports/history, position papers epidemiological data and the large body of evidence acquired thereby, has been utilized, clinically, to arrive at a consensus statement, based on internationally accepted and followed criteria. This approach is known as Evidence-based Medicine (EBM), and is meant for improvements in the diagnosis and/or treatment of human diseases (Chalmers 1993). Evidence-based Research (EBR) involves the systematic evaluation of the scientific literature, which can potentially lead to Complementary and Alternative Medical approaches (Chiappelli et al. 2006). With respect to oncology, the underlying tumor heterogeneity, due to multiple etiological (genetic, epigenetic, stochastic,

environmental) contributing factors, coupled to the complexity conferred by the stromal micro-environment warrants development of Gene Expression Signatures (GES) which would help in better patient stratification, with its ultimate goal being “designer therapy” or personalized medicine (Bild et al. 2006; Potti et al. 2006). The current approaches for GES development currently involves utilization of the large body of existing gene expression profiling data (microarray) from biopsies as well as resected surgical specimens and the correlation of such data with samples obtained, by relatively non-invasive means, from biological fluids like blood and saliva with the understanding that any approach would have to be correlated with assays that are “gold standards”. In addition, the development of GES requires high-throughput methods. Reproducible correlation of such signatures, using *in vitro* approaches would serve enormously to speed up the process. However, this approach should be robust,

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scalable, sensitive, and specific and lends itself to miniaturization.

Clinicians rely on the use of information from Immunohistochemistry (IHC) as well as Enzyme linked Immunosorbent Assay (ELISA). However, in certain cases, molecular diagnostic approaches (at the genomic, transcriptomic and proteomic level) may be needed. For e.g., the different forms of the Epidermal Growth Factor Receptor (EGFR) may be detected in one experiment as demonstrated by the multi-layered Western Blotting technology in HNSCC cell lines, which can be potentially applied to analyze proteins obtained from biopsy specimens and cell lines (Patel et al. 2005) which, in turn, can pave the way for the development and validation of cost-effective approaches (Tabone et al. 2006). An example of “a molecular signature” is the G:C to T:A transversion in codon 259 of the TP53 gene associating aflatoxin exposure and hepato-cellular carcinoma in a Chinese study, using *ex vivo* approaches (Stern et al. 2001). Microarray technology can be used to develop GES, based on drug sensitivity in *in vitro* model systems, and this information may be applied to identify those patients who might benefit from treatment. In this regard, multi-gene signature indicative of an epithelial to mesenchymal transition (EMT) was identified as a determinant of insensitivity to erlotinib through both supervised and unsupervised gene expression approaches (Yauch et al. 2005). A common Proteomics approach, 2D-Gel Electrophoresis (2D-GE), has been used for protein profiling using primary renal cell lines and concordance has been obtained with other classical methods like Western Blotting and Immunohistochemistry. Identification of novel molecules by 2-DE, included up-regulation of several proteins involved in actin cytoskeleton organization such as radixin and moesin, two members of the septin family, and the actin bundling protein, lending more credence to the approach of using cell lines-based GES development (Craven et al. 2006). Hence, a combination of genomic, transcriptomic and proteomic approaches, based on samples from *ex vivo/in vitro* means, would improve the predictive power of the GES, with the obvious fact that it would have to go through the clinical validation study.

SAMPLING CONSIDERATIONS

Validation and evaluation of GES will be to

ascertain their sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) (Buckhaults 2006) before it can be translated from the benchside to the chairside. Hence, patient selection and stratification (for longitudinal and cross-sectional), is of paramount importance to develop, validate and utilize such GES for biomarkers that, in turn, can speed up the process of drug development; identify responders and non-responders as well as improve now the efficacy and therapeutic index. In this regard, development of novel biomarkers would serve to better handle research questions, regarding adverse/hypersensitivity reactions as well as trying to salvage drugs by identifying a subset of the human populations for which the drug may be very effective (Simon and Wang 2005). In this regard, choice of an appropriate test and training set may be critical for the validation of well characterized biomarkers like NF-kB, that can predict outcome of a therapeutic intervention as well as patient stratification into high-risk and low risk groups (Chung et al. 2006). While any clinical study has its own strength and limitations, it is considered that the classical case-control study (Ranganathan et al. 2004) would be the best approach to stratify responders and non-responders (Simon and Wang 2005) and can be used for testing the genomic signature (Simon and Wang 2005).

While the predictive power has been reported in several parts of the world using (“predictive gene lists”), distinct sets of genes have been reported for similar tumors. Hence, sample size considerations; coupled to global collaboration, using standardized cross-validated protocols would serve to enormously save time (Ein-Dor et al. 2006). In this regard, it is extremely important to remember that the currently available neural networks may represent an algorithm written keeping in mind the entire gamut of clinical features while the GES, from a sample, may perform have to be based on the clinical features seen in the sample (Simon and Wang 2005).

EX VIVO / IN VITRO APPROACHES FOR WOUND RESPONSE SIGNATURES

Since cancer is considered by some to be “wound healing gone awry”, studies involving the commonalities in mechanisms between aberrant wound healing and cancer progression have been performed globally (cf. Schottenfeld

and Beebe-Dimmer 2006; Dvorak 1986). This is especially so because the medical literature, based on epidemiological studies, is replete with classical examples like chronic inflammation in the cirrhotic liver of alcoholics being associated with hepato-cellular carcinoma (Seitz and Stickel 2006) and chronic gastric inflammation with gastric cancer (Schottenfeld and Beebe-Dimmer 2006). The commonalities between aberrant wound healing and malignancy warranted scientists globally to evaluate critically the proteins and their variants that would be at the crossroads of inflammation and malignancy (Karin and Greten 2005; Kornman 2006) with the knowledge that Cancer involves aberrations in gene(s) and/or gene products associated with proliferation, differentiation, apoptosis, angiogenesis and invasive potential and this information should also be coupled to data obtained by human cancer cell lines (*in vitro*) (Kondoh et al. 2006; Jeon et al. 2004). In this context, elegant experiments have been made possible by the availability of RNAi technologies (stable RNA interference) which would permit the specific knock-down of a gene in the cellular context, thereby permitting the evaluation of its role and/or its potential in terms of its prognostic significance (Sun et al. 2007). This *in vitro/ex vivo* approach could add prognostic value to the existing wound response signatures (Liu et al. 2006). In addition, correlation of a gene, within a gene set; or overlapping/non-overlapping gene sets (Massague 2007) with the outcome; independent of the other(s) can increase the predictive power with a concomitant decrease in cost (Adler and Chang 2006). While this approach may not be all encompassing, it define “a minimal gene set” that has the same predictive potential. In this context, the Adler and Chang approach has tremendous potential since the 512 gene response set, related to cell growth, matrix remodeling, cell motility and angiogenesis was utilized and reduced to two genes (MYC and CSN5). In the same paper, transfection of these two genes into normal mammary epithelial cells mimicked the transcriptional profile of a “wound response signature” seen in “authentic human tumors” (Adler and Chang 2006). Corroborative evidence for this type of signature was obtained by the stimulation of normal fibroblasts (*ex vivo*) by serum and subsequent transcriptional profiling and the results obtained thereby, were compared with those from tumors (Chang et al.

2004). As an example, an aberrant localization of FGF-2 and FGFR2 in the epithelial layers with increasing dysplasia, suggests an increased mitotic potential of high-level cells. This information with a concomitant loss of FGF-1 seems to indicate its important role in the de-differentiation seen in dysplasia and squamous cell carcinoma. However, a Japanese study has shown that well-differentiated and poorly-differentiated cancer cells were positive for FGF-1 and FGF-2 with high frequency and intensity as compared to normal oral epithelium (Myoken et al. 1994). On the other hand, over-expression of EGFR followed by c-myc overexpression as well as consecutive reactivation of telomerase, following induction of EGFR, sufficed to transform oral epithelial cells, truly mimicking the development of the corresponding human disease (Goessel et al. 2005). Hence, modelling of such complex mechanisms would necessarily involve co-culture experiments, by which the contribution of the stromal micro-environment may be better evaluated (Croonquist et al. 2003). In another experiment, using an *in vitro* model of mouse mammary epithelia cells (31EG4-2A4), the mechanistic role of pro-inflammatory cytokine (TNF- α) in the loss of cell-cell contact, mediated by integrins, like β 1-integrin, and MMPs, like MMP-9, and invasive properties based on the cytokine’s ability to cause 3D scattering, which, otherwise would form compact spheroidal colonies (Montesano et al. 2005).

KEY MOLECULAR BIOLOGICAL TECHNIQUES FOR *EX VIVO* / *IN VITRO* APPROACHES

These aforementioned experimental designs, would corroborate other approaches like using exfoliated cells from the oral mucosa to perform molecular biological experiments-Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR). This approach is attractive due to the fact that samples can be obtained by relatively non-invasive means. However, this method also documents the inter-individual variability in mRNA levels, as has been reported in other studies (Spivack et al. 2004) as well as Multiple Ligation Probe Amplification (MLPA). Using MLPA, 20 –50 ng of DNA was found to be sufficient to measure gains and losses at 42 different chromosomal locations in one PCR-reaction and the samples could be obtained by

an easily performed brushing technique (Bremmer *et al.* 2005). Approaches like Differential Display-PCR (DD-PCR) (Liang and Pardee 1992) using DNA/cDNA probes, which lends itself to the identification of novel genes and micro-array technology which permit the simultaneous expression of differentially expressed genes were combined and modified as the “comprehensive validated differential display”. This experimental modification, has led to a 73% concordance of DD sequences with human proteins when this method was adopted. Furthermore, the genes enriched included those involved in processes, relevant to the processes of carcinogenesis, like cell motility, protein synthesis, stress and immune responses, cell death, cell cycle, cell proliferation and/or maintenance of transport. (Carles *et al.* 2005). Other approaches like qPCR-based proteomic mapping (phage display bio-panning), performed in lab animals, seems to have potential and may lend itself to the identification of epitopes in individual cells and cell types (Ballard *et al.* 2006). However, more studies, like these are warranted, with a large sample size, using human samples from a wide variety of human tissues.

PROTEOMICS-BASED APPROACHES

Proteomic approaches using a wide variety of methods like Matrix Assisted Laser Desorption/Ionization-Time of Flight-mass spectrometry (MALDI-TOF) as well as Surface-enhanced Laser Desorption/Ionization (SELDI)-based approaches coupled to liquid chromatography. For e.g., MALDI-TOF has been used to develop a proteomic map of adult human normal fibroblasts in culture and has also been used for the evaluation of membrane-bound and secreted proteins. This approach also included the comparison of gel and MS data (Boraldi *et al.* 2006). SELDI-TOF has been used to identify novel biomarker in pluripotent stem cells and their differentiation status (Hayman and Przyborski 2004).

Certain Low Molecular Weight Markers (< 15 kDa) complexed with albumin, hitherto considered to be “biological trash” represent breakdown products in blood following enzymatic degradation and may have diagnostic and/or prognostic potential. They may not be detectable using current methods of protein preparation for a routine Western Blotting analysis, with

conventional protease inhibitors, and hence, possibly suppress endogenous biomarkers (Liotta and Petricoin 2006). Such markers, as well as low abundance, high molecular weight markers (390 kDa –BRCA2) (Lowenthal 2005) have resulted in a new era of peptidomics to add and enhance the predictability of proteomics-based GES (Liotta and Petricoin 2006).

DATABASE

This data set can then be incorporated to improve existing bio-informatic approaches as well as develop novel *in silico* methods. In this regard, several databases like Max Planck Unified Proteome Database (MAPU) (Zhang *et al.* 2007); CYCLONET (Kolpakov *et al.* 2007); Integrated Transcriptome Array and Clinical Analysis (ITTACA) (Elifilali *et al.* 2006), PEPPer (Jaffe *et al.* 2006) ARACNE (Margolin *et al.* 2005) etc have been developed. In the case of MAPU, proteomes, being dynamic in nature, results obtained from cell culture systems can add to the information obtained from other sources like homogenized tissue as well as body fluids (blood, saliva etc). In this regard, the choice of cell type is of important. For e.g., HeLa cells could be used due to ease of transfectability. However, protein-protein interactions would require the binding partner to be expressed in the experimental *in vitro* model. On the other hand, corroborative evidence, integrating genomic and proteomic data, of this nature would enable us to better understand the complex networks involving transcription factors, binding motifs as well as co-expressed genes (Zhang *et al.* 2007). Cyclonet (CYCLONET) database serves to integrate information about cell cycle regulation from a wide variety of experimental methods in the form of diagrams, models, statistical interpretation of micro-array data as well chemo-informatics data (Kolpakov *et al.* 2007). The approach taken by the developers of ITTACA, in combining expression profiling data with the clinical information along with a user-friendly web interface as well as comparison with data stored in other repositories through the development of a data import and export functionality through Microarray Gene Expression Markup Language (MAGE-ML) (Elfilali *et al.* 2006). Platform for Experimental Pattern Recognition (PEPPER) utilizes several algorithms including Land Mark Matching (utilizing historical data acquired by

different strategies) and Peak Matching. The latter algorithm, builds on the former, by recognizing identical molecules from separate experiments using a clustering approach (Jaffe et al. 2006). Last, but not least, a direct off shoot of *in vitro* data development would be an algorithm, like ARACNE, where complex regulatory networks, which would be different in the various cellular contexts, can be elucidated based on its ability to correlate micro-array data with functional mechanisms (Margolin et al. 2006).

CONCLUSIONS

In conclusions, *in vitro* approaches should be correlated and subsequently integrated with other approaches involving clinical findings, genomic data (SNPs, epigenetic factors etc), micro-array results, proteomic and metabolic data and this should be coupled with bio-informatic strategies to further evaluate potential conflicting predictors of risk (West et al. 2006). Extension of the “prognosis space” by developing overlapping gene sets can enhance the predictive value of GES (Massague 2007) using a variety of *ex vivo* and *in vitro* approaches. However, inter-individual variability and other confounding factors like (age, gender, lifestyle, diet, occupation, changing risk profiles due to region-specific environmental influences) reiterates the imperative necessity for a sound experimental design, with the research scientist interfacing with the clinician/oncologist in terms of inclusion/exclusion criteria and clinical correlates for a better interpretation of the genomic classifier obtained by *in vitro/ex vivo* data methods.

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